NEW ASSOCIATIONS BETWEEN *DELADENUS* NEMATODES, THEIR *SIREX* HOSTS, AND FUNGAL SYMBIONTS

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NEW ASSOCIATIONS BETWEEN *DELADENUS* NEMATODES, THEIR *SIREX* HOSTS, AND FUNGAL SYMBIONTS

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The invasive woodwasp, *Sirex noctilio*, has significant negative impacts on pine trees. Since the discovery of established populations of *S. noctilio* in New York State and Ontario in 2005, a parasitic nematode, *Deladenus siricidicola*, has been considered for release for biological control in the United States. North American *Sirex* control proves to be complicated, due to the presence of native species of *Sirex* which are not considered to be pests, different species and isolates of the obligate fungal symbiont of *S. noctilio, Amylostereum areolatum*, and the presence of different species and strains of *Deladenus* nematodes. Nematodes in the genus *Deladenus* associated with siricids have two different forms: a mycophagous form that feeds on the *Sirex*-symbiotic fungi and a form that parasitizes *Sirex* and is transferred from tree to tree during oviposition by *Sirex*. I evaluated the ability of *D. siricidicola* (Kamona strain) to reproduce when feeding on different isolates of *A. areolatum* and found that nematode populations persisted on all isolates tested. One of the slowest growing fungal isolates, ScyME, produced the most nematodes when the fungus was given five and ten days of growth prior to nematode inoculation, while the fastest growing fungus, Aussie, never produced the most nematodes. Although nematodes in all treatments produced eggs, *D. siricidicola* populations were unable to replace themselves when feeding on the fungal isolate SedDF. In another study, a culture of *Deladenus* nematodes established from a native *Sirex nigricornis* was identified as *Deladenus proximus*

using molecular and morphometric techniques. I compared the ability of *D. proximus* and *D. siricidicola* (Kamona) to reproduce when feeding on native and invasive isolates of *Amylostereum* fungus. *D. siricidicola* were able to reproduce on all isolates of *A. areolatum* tested, but reproduced poorly on the *A. areolatum* isolate they would be most likely to encounter in northeastern North America, should the nematode be released. *D. proximus* were able to reproduce well on both *A. chailletii* and *A. areolatum*, despite prior evidence suggesting only *A. chailletii* is a suitable food source, leading to the suggestion that this native nematode should be evaluated for its ability to parasitize and sterilize *S. noctilio*. To study phylogenetic relationships among native *Deladenus* spp. in the northeastern United States and the Kamona strain of *D. siricidicola*, three genes (mtCO1, LSU, and ITS) from nematodes extracted from parasitized *Sirex* spp. collected inside and outside of the range of *S. noctilio* were analyzed. Results showed each *Sirex* species has its own associated *Deladenus* parasite. This study provided evidence that *D. proximus* can parasitize *S. noctilio*, and that *D. siricidicola* can parasitize *S. nigricornis*, indicating potential for non-target impacts of a biological control program using *D. siricidicola* against *S. noctilio*. In another study, I investigated a hypothesized role reversal wherein fungal hyphae invade and kill nematode eggs. *D. siricidicola* eggs were exposed to multiple isolates of *A. areolatum* to quantify the number of eggs lost to fungal invasion. *A. areolatum* and *A. chailletii* were observed via a combination of cryogenic scanning electron microscopy and fluorescence microscopy for their ability to parasitize both eggs and adults of *D. siricidicola* and *D. proximus*. This study reports the first evidence of a Basidiomycete destroying nematode eggs, as well as a novel trapping mechanism used to capture and parasitize two species of adult female *Deladenus*.

BIOGRAPHICAL SKETCH

Erin Morris was born on the 16th of July, 1983 in Leesburg, Ohio, to Heidi and Doug Morris. She graduated from Fairfield Local High School in 2001. While earning her Bachelor's of Science degree at the Ohio State University, she began taking Entomology courses, fell in love with the subject, and has not looked back since. Following her 2005 graduation, she joined Dr. Parwinder Grewal's lab at the Ohio State University. There, she completed her Master's of Science degree in Entomology, for which she studied the biological control of Japanese beetles using entomopathogenic nematodes and fungi.

In 2009, Erin joined Dr. Ann Hajek's invertebrate pathology lab at Cornell University as a Ph.D. student. Her project has revolved around the invasive pine pest, *Sirex noctilio*, and its nematode biological control agent, *Deladenus siricidicola*. Following graduation, Erin intends to continue work with beneficial nematodes and pursue a post-doctoral position with Dr. Jørgen Eilenberg at the University of Copenhagen in Denmark.

Dedicated to Heidi, Doug, and Joel. We move as a unit.

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I would not have gone into science without my mom, Heidi Morris. Thanks to her I grew up with *Discover Magazine, Scientific America*, and Carl Sagan's *Pale Blue Dot*, and developed an early interest in the sciences. My dad, Doug Morris, taught me the joy of flipping over rocks with a jar in hand. Together my parents have been with me every step of the way, providing endless support. Thank you guys.

I thank Dr. Jerry Pausch for providing scholarships to students from my home county for studying science at The Ohio State University. Jerry's given me so much support throughout my entire undergraduate and graduate career. He also loves roller coasters at amusement parks and Buckeye football, and he's taken me to enjoy both of those things on numerous occasions. His devotion to making science education accessible to high school students is immense, and I hope to pay it forward.

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Literature Review

1.1 *Sirex noctilio* as a pest

The invasive woodwasp *Sirex noctilio* Fabricius (Hymenoptera: Siricidae) has caused significant negative impacts on pine trees in the Southern Hemisphere for nearly one hundred years. Native to Eurasia and northern Africa, *S. noctilio* was first noted outside of its native range in New Zealand in approximately 1900, although it was not a considerable pest at that point (Miller and Clark, 1935). *S. noctilio* larvae predominantly develop in pine trees and, while pines are native only to the Northern Hemisphere, they have been planted in plantations in many areas of the temperate Southern Hemisphere. By 1952, *S. noctilio* was discovered in pine plantations in Tasmania, and by 1961 it had made its way to mainland Australia. The wasp continued to spread across the Southern Hemisphere, establishing in Uruguay, Argentina, and Brazil in the 1980's, in South Africa in 1994, and in Chile in 2001 (Hurley et al., 2007; Beèche et al., 2012). Although *S. noctilio* is considered a secondary pest of pines in its native range (Spradbery and Kirk, 1978; Furniss and Carolin, 1977; Madden, 1988), by 1926, it was considered a serious pest of pine trees in New Zealand (Cameron, 2012), and to this day it remains a major pest of pines in plantations across its introduced range in the Southern Hemisphere (Rawlings 1948; Hurley et al. 2007; Ryan and Hurley, 2012).

S. noctilio was first collected in North America in 2004 in New York State, and in Ontario in 2005 (Hoebeke et al., 2005; de Groot et al., 2006). In the United States, *S. noctilio* is

currently established in New York, Pennsylvania, Vermont, Michigan, Ohio, Connecticut, and New Jersey (NAPIS, 2013).

Adult *S. noctilio* females are unique among species of *Sirex* woodwasps in their ability to kill healthy pine trees. They are able to do so due to a combination of a symbiotic wood rot fungus, *Amylostereum areolatum* (Fries) Boidin, and a phytotoxic venom, both of which are injected into the tree during oviposition. Together, the venom and fungus lead to lethal wilt of the pine tree, and the fungus subsequently grows throughout the wood (Coutts, 1969a; Coutts, 1969b). In situations where pines are stressed, such as due to drought, *S. noctilio* can reach outbreak populations that can be devastating. For example, in the 1980s, such an outbreak population occurred in southeastern Australia and up to 1.8 million Monterey pines were killed (Haugen and Underdown, 1990).

Control of *S. noctilio* in the Southern Hemisphere includes two main strategies. The first strategy, based on the woodwasp's preference for stressed trees, is to reduce stress in pine plantations via good silvicultural practices such as stand thinning. The second main strategy is biological control. Numerous parasitoid wasps have been released and established across the Southern Hemisphere, including species of *Rhyssa* and *Megarhyssa* (Hymenoptera: Ichneumonidae) and *Ibalia* (Hymenoptera: Ibaliidae) that kill *S. noctilio* larvae (Hurley et al., 2007; Cameron, 2012). The major biological control agent used against *S. noctilio*, however, is a parasitic nematode, *Deladenus siricidicola* Bedding (= *Beddingia siricidicola*) (Tylenchida: Neotylenchidae), which sterilizes adult *S. noctilio* females. This nematode is commercially produced in Australia and it is released in an inoculative fashion in Australia, South Africa, and parts of South America (Hurley et al., 2007).

The *S. noctilio* invasion in North America is unique in that, unlike when it was introduced to the non-native pine plantations of the Southern Hemisphere, in North America this invasive is within the native range of pine trees (Bedding, 2009). The North American pines host a rich community of native species of *Sirex*, as well as associated hymenopteran parasitoids and nematode parasites. Thus far, no *S. noctilio* control program is in place in North America, although the release of *D. siricidicola* nematodes for biological control is under consideration.

1.2 Biology of *Sirex noctilio*

The life cycle of *S. noctilio* is intertwined with that of its symbiotic white rot fungus, *Amylostereum areolatum*. Fragments of the fungus, called arthrospores, are carried in adult females within a pair of internal organs called mycangia at the base of the ovipositor. Arthrospores are injected into the host pine tree during oviposition, as well as phytotoxic venom. The fungus grows throughout the wood, drying it to create a suitable environment for developing *S. noctilio* larvae (Madden and Coutts, 1979). Additionally, the fungus provides nutrients and enzymes necessary for larval development, and larvae cannot develop into adults in its absence.

Developmental time to adulthood usually takes one year, although this may take up to three years for a single generation in colder climates (Ryan and Hurley, 2012). Adults emerge from early summer until autumn, with males generally appearing first. Upon emergence, mating occurs and females search for suitable pine hosts for oviposition. Female *S. noctilio* are pro-ovigenic, so at eclosion they have a full complement of from 30-450 eggs. The number of eggs in a single adult female is related to body size, with larger females containing more eggs (Madden, 1974; Kroll et al., 2013).

Stressed pine trees are preferred for oviposition, and to assess the health of a pine, the female drills into the tree with her ovipositor to survey osmotic pressure of the phloem sap. If a host tree is found to be suitable up to four drills are made at different angles (Coutts and Dolezal, 1969). Eggs and phytotoxic venom are deposited into each drill, with a final drill containing only arthrospores and phytotoxic venom. If a host tree is considered unsuitable, the female may instead inject phytotoxic venom and fungal arthrospores but not an egg. Thus, even vigorous trees may be attacked (Madden and Coutts, 1979).

1.3 Use of *Deladenus siricidicola* to control *Sirex noctilio*

The nematode *D. siricidicola* was first found parasitizing *S. noctilio* in New Zealand in 1962 (Zondag, 1962), where it was thought to have been transported along with the invading *S. noctilio.* In the 1960s, a worldwide search for natural enemies of siricid woodwasps was conducted, resulting in the discovery of several hundred strains of seven species of *Deladenus* nematodes (Bedding and Akhurst, 1978; Bedding, 1974). Because these nematodes have a mycophagous as well as a parasitic life stage, laboratory cultures were established on potato dextrose agar cultures of either *Amylostereum areolatum* or *A. chailletii*, depending on which fungal species was associated with the appropriate siricid host within which each nematode strain had been found.

Deladenus siricidicola was eventually chosen as a biological control agent of *S. noctilio* based on several criteria. First, although the nematode was able to parasitize *S. noctilio*, it did not parasitize the hymenopteran parasitoids of *S. noctilio*. Second, *D. siricidicola* was able to reproduce when feeding on *S. noctilio's* symbiotic fungus, *A. areolatum*. All other tested species

of *Deladenus* fed exclusively on *A. chailletii*, with the exception of *D. wilsoni*, which fed on both species of fungus. *D. wilsoni* was found to parasitize hymenopteran parasitoids of *S. noctilio*, however, and was therefore eliminated from consideration (Bedding and Akhurst, 1978). Once *D. siricidicola* was chosen as a candidate for biological control, the Sopron strain, from Hungary, was selected for several reasons. First, some *D. siricidicola* strains were found to be non-sterilizing in that they were unable to penetrate *S. noctilio* eggs and thus sterilize the adult females, despite their ability to parasitize the wasp (Bedding, 1972). The Sopron strain, however, did lead to sterilization of high percentages of *S. noctilio* females emerging from trees. Additionally, parasitism with this strain did not reduce the size of *S. noctilio*, allowing parasitized adults to disperse farther and better propagate the nematode (Bedding and Iede, 2005).

Releases of *D. siricidicola* Sopron strain were made in the Southern Hemisphere with varying success until it was discovered in 1987-1900 to have attenuated and lost virulence due to repeated lab culture (Haugen and Underdown, 1993; Bedding and Iede, 2005; Bedding 2009). At that point, *D. siricidicola* was reisolated from parasitized *S. noctilio* in the Kamona forest, in Tasmania. This strain was renamed *D. siricidicola* Kamona, and is the strain that has been used for biological control of *S. noctilio* in the Southern Hemisphere to date. Hurley et al. (2007) provide an excellent summary of successes and failures in the control of *S. noctilio* by *D. siricidicola* in the Southern Hemisphere.

1.4 Biology of *Deladenus siricidicola*

Deladenus siricidicola has two life stages, including a free-living form and a parasitic form. The free-living form is mycophagous and feeds on *A. areolatum* and the parasitic form invades larval *S. noctilio*. Although the nematodes parasitize male and female larvae, males are considered a dead end host. If the nematodes within a female host penetrate *S. noctilio* eggs during pupation of the woodwasp, this leads to sterilization of adult females (Bedding, 2009).

The mycophagous form of *D. siricidicola* eats the hyphal tips of *A. areolatum* growing throughout a pine tree. Nematodes in this form can undergo 20-30 generations per year and disperse throughout the tree to wherever the fungus grows. Development into the parasitic form is induced by the presence of host cues such as lower pH and increased CO_2 levels (Bedding 1993). This causes nematodes to develop into parasitic adult males and females. Parasitic adults mate, and then females invade *S. noctilio* larvae. Parasitic females inside of a larval host shed their outer cuticles to expose extensive microvilli through which they passively absorb food from host hemolymph. The reproductive systems of parasitic females do not fully mature until the host pupates. The fully mature parasitic female lays eggs which hatch, and in a female host the juveniles subsequently migrate to the host ovaries. For host sterilization to occur, the juvenile nematodes must migrate into host eggs prior to the hardening of the chorions. Otherwise, juvenile nematodes are associated with, but not within, host eggs. In either case, nematodes are transferred to a new location when the adult *S. noctilio* emerges and oviposits in a new tree. Unlike the two-week generation time of the mycophagous form, a single generation of the parasitic form may take from 1-3 years to complete (Bedding, 1972).

1.5 Control of *Sirex noctilio* in North America using *Deladenus siricidicola*

Since the discovery of an established population of *S. noctilio* in the northeastern United States and Ontario in 2005, pest risk analyses have been conducted. *S. noctilio* is considered a high risk pest in many regards, and the release of *D. siricidicola* Kamona strain has been considered for its control (Borchert et al., 2007). However, the ecological system surrounding *S. noctilio* in the Southern Hemisphere, where pine trees are introduced, is markedly different from *S. noctilio* in North America, where pine trees are native. North American *S. noctilio* control proves to be much more complicated, with factors not present in the Southern Hemisphere; namely, native species of *Sirex* which are not considered to be pests, different species and isolates of the obligate fungal symbiont of *S. noctilio*, and the presence of different species and strains of *Deladenus* nematodes (Williams and Mastro, 2010; Williams et al., 2013; Nielsen et al. 2009; Hajek et al. 2013; Yu et al., 2009). To understand how these new potential interactions could affect a biological control program for *S. noctilio* using *D. siricidicola*, we must first delve into the individual life histories of wasp, fungus, and nematode, from which point we can begin to understand their interactions with one another, and finally, be able to infer new associations that could arise.

1.6 Investigating interactions between invasive and native *Sirex* communities in pine

Chapter 1 reviews the general biology of *Sirex* and *Deladenus* and discusses the potential use of *D. siricidicola* for biological control in North America. Chapters 2 and 3 investigate the interactions between *Deladenus* nematodes and *Amylostereum* fungi. The studies include

measuring the reproduction of two *Deladenus* species feeding on species and isolates of *Amylostereum* which are currently found in North America. Based on the results, suggestions are made for appropriate fungal isolates to be considered for mass production of *D. siricidicola* in the United States. Additionally, due to the discovery of a wider fungal food range for a native North American nematode species, *D. proximus*, Chapter 3 highlights the potential for non-target effects as a result of applying *D. siricidicola* to trees that are co-inhabited by both invasive *S. noctilio* and native *S. nigricornis.*

Chapter 4 is predominantly a phylogenetic study which describes the different *Deladenus* nematodes currently parasitizing eastern North American *Sirex* woodwasps, finding that each *Sirex* species in the range sampled has its own corresponding nematode parasite. At the same time, this study shows that cross-infectivity of nematode species occasionally occurs between *Sirex* woodwasps, which further suggests the potential for non-target effects towards North America's native *Sirex*.

While prior chapters focus on the reproduction of *Deladenus* species on different species and isolates of *Amylostereum*, Chapter 5 examines the ability of *Amylostereum* fungi to use the nematode as a food resource in a reversal of roles. This study provides evidence of the first Basidiomycete fungus reported as being a nematode egg parasite. The number of *Deladenus* eggs failing to result in juveniles were quantified and compared to the number of eggs when nematodes were not exposed to *Amylostereum*. Additionally, cryogenic scanning electron microscopy and fluorescence microscopy were used to gain visual insight into this reversal of predatory roles.

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Chapter 2

Variability in growth of *Deladenus siricidicola* on isolates of the white rot fungus *Amylostereum areolatum*[1]

2.1 Abstract

Deladenus siricidicola nematodes are used extensively for biological control of *Sirex noctilio* in the southern hemisphere. One morph is parasitic on *S. noctilio* and another feeds on the white rot fungus *Amylostereum areolatum* and is used for mass production of the nematode. To examine potential effects of isolates of *A. areolatum* found in North America on *D. siricidicola* in a biological control program, we first compared the growth of four isolates of *A. areolatum* on several types of artificial media. We then evaluated the ability of *D. siricidicola* to survive and increase on five isolates of *A. areolatum* and found that nematode populations persisted on all five isolates. One of the slowest growing fungal isolates, ScyME, produced the most nematodes when the fungus was given five and ten days of growth prior to nematode inoculation, while the fastest growing fungus, Aussie, never produced the most nematodes. Although nematodes in all treatments produced eggs, *D. siricidicola* populations were unable to replace themselves when feeding on the fungal isolate SedDF. The differential ability of *D. siricidicola* to persist on

[1]Morris, E.E. [a], Jimenez, A. [a], Long, S.J. [a], Williams, D.W. [b], Hajek, A.E. [a], 2012. Variability in growth of *Deladenus siricidicola* on strains of the white rot fungus *Amylostereum areolatum*. BioControl 57, 677-686.

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different isolates of *A. areolatum* found in North America could affect multiple aspects of a biological control program to control *S. noctilio*.

Keywords: biological control; woodwasp; nematode; *Sirex noctilio*; *Sirex*; invasive species

2.2 Introduction

The nematode *Deladenus* (=*Beddingia*) *siricidicola* Bedding (Tylenchida: Neotylenchidae) has an extraordinary dual lifestyle which alternates between fungal-feeding and insect-parasitic (Bedding 2009). The fungal-feeding form lives in trees and feeds on a white rot fungus, while the parasitic form infects *Sirex* woodwasps, which then carry the nematode to a new tree. Because parasitism often results in sterilization of the female woodwasp, the nematode has been used extensively for biological control of the invasive pest *Sirex noctilio* F. (Hymenoptera: Siricidae) in the southern hemisphere (Hurley et al. 2007).

The woodwasp *S. noctilio* is native to Eurasia and northern Africa and in these regions it is not considered a pest, but it has caused extensive damage since its introduction to New Zealand, Australia, several South American countries and South Africa (Hurley et al. 2007). *Sirex noctilio* principally infest pine (*Pinus* spp.) trees, into which ovipositing females deposit the symbiotic white rot fungus *Amylostereum areolatum* (Fr.) Boidin (Russulales: Amylostereaceae) during oviposition. *S. noctilio* larvae develop while feeding on *A. areolatum*-decayed wood and do not survive unless the fungal symbiont is present (Madden and Coutts 1979). An individual from an established population of this woodwasp was first collected in New York near Lake Ontario in 2004 (Hoebeke et al. 2005), and in 2005 it was discovered in Ontario, Canada (de Groot et al. 2006). In the United States, *S. noctilio* spread throughout New York and

into Pennsylvania by 2006, to Vermont and Michigan by 2007, to Ohio by 2008, and to Connecticut by 2010 (USDA, APHIS 2010).

Female *S. noctilio* that are infected with the parasitic form of *D. siricidicola* are sterilized, but disperse their nematode-filled eggs to new trees, also transporting the fungus needed to sustain the mycophagous form of the nematode (Bedding 2009). The nematodes can complete multiple generations in the mycophagous phase; based on this fact, mass production of *D. siricidicola* in Australia for biological control uses the fungal-feeding form. The *A. areolatum* isolate used for mass production has remained in use since its collection from Sopron, Hungary in 1968 (R. A. Bedding, pers. comm.).

While *Deladenus* species are not particularly insect-host specific, they are fungus specific (Bedding and Akhurst 1978), and although feeding may occur on a given fungus, reproduction may be restricted. Pillai and Taylor (1968), when studying four fungal-feeding nematodes, including *Neotylenchus linfordi*, found that of the ten fungi studied, none of them repelled the nematodes. When they assessed the total nematodes produced on the fungal plates, however, they found that the fungi differed in host suitability, ranging from excellent to poor. Indeed, Hurley et al. (2007) listed fungal-nematode incompatibility as a possible reason for inadequate control of *S. noctilio* in the KwaZulu-Natal region of South Africa. Slippers et al. (2001) speculated that such an incompatibility between nematode and fungal isolate could affect nematode feeding and reproduction when nematodes are introduced to new areas. Given that fungal isolate may affect nematode reproduction and that native North American *Sirex* spp. carry different isolates of *A. areolatum*, nematode growth on different fungal isolates could significantly impact a biological control program involving *D. siricidicola*.

Biological control of *S. noctilio* in the southern hemisphere (Hurley 2007; Bedding 2009; Borchert et al. 2007) has provided a good framework for such an effort in the northeastern United States and Canada. However, several factors complicate direct adoption of the approach used in the southern hemisphere. Unlike the southern hemisphere, where pine trees have been introduced as a managed crop, pines are native to North America (Hurley et al. 2007). Likewise, in the southern hemisphere, the invasive *S. noctilio* is the only siricid, whereas North America hosts several native species of *Sirex* woodwasps that are not considered to be pests (Smith and Schiff 2002; Slippers et al. 2003). Some native North American *Sirex* carry *Amylostereum chailletii* (Fr.) Boidin as a symbiont, although at least one North American *Sirex, Sirex 'nitidus'*, is now thought to carry *A. areolatum* naturally at least some of the time (Nielsen et al. 2009). The isolates of *A. areolatum* found in native *Sirex* spp. are not the same that are found in *S. noctilio*, based on genetic sequencing data.

With the potential to release *D. siricidicola* in North America, we were interested in whether *D. siricidicola* will feed on the isolates of *A. areolatum* present in North America, and we hypothesized that isolates of *A. areolatum* with faster growth rates would produce more *D. siricidicola*. First, we chose four fungal isolates of *A. areolatum*, including the isolate used for mass production of *D. siricidicola* in Australia, and determined the relationship between fungal isolate and growth rate using different media. We compared the media used for mass production of *A. areolatum* with types of media used in other studies: Hagem agar (Stenlid 1985), potato dextrose agar amended with thiamine, and green bean agar (Thomsen and Koch 1999). Next, we grew five isolates of *A. areolatum* on one type of medium and then inoculated the fungal cultures with *D. siricidicola* to investigate the relative increase of the nematodes on the different isolates of fungus.

2.3 Materials and methods

2.3.1 Fungal isolates

The geographic and host origins of the five isolates of *A. areolatum* included in this study are listed in Table 2.1. Four of the isolates were obtained from *Sirex* collected in North America and these were chosen to represent the diversity of *Sirex* hosts and of genotypes present in North America, based on intergenic spacer regions (IGS) (Nielsen et al. 2009). One of these isolates is thought to be native (ScyME) and three are thought to be introduced (OtisAa, Gr94-1, and SedDF). The fifth fungal isolate (Aussie) was obtained from *Sirex juvencus* (L.), collected in Hungary and maintained in culture since 1967-1968. This isolate was obtained from Ecogrow Environment (Queanbeyan, N.S.W., Australia) where it is used for mass production of the *S. noctilio* biological control agent *D. siricidicola*.

Table 2.1. Isolates of *Amylostereum areolatum*.

Isolate ID	Isolation from	Original host	Date Isolated	IGS Type
Aussie	Sopron, Hungary	*S. juvencus*	1967-1968	BDF
OtisAa	Oswego County, NY	*S. noctilio*	Jan 2006	D
ScyME	Waldo County, ME	*S. nitidus*	13 Sep 2007	BE
Gr94-1	Fulton County, NY	*S. noctilio*	19 Feb 2008	BD
SedDF	Oswego County, NY	*S. edwardsii*	19 Sep 2007	D

[1] Intergenic spacer region (IGS) isolates as reported in Nielsen et al. (2009).

2.3.2 Nematode strain

The strain of *D. siricidicola* used for studies was obtained from Ecogrow Environment where it is mass produced for biological control. The nematodes were obtained from Ecogrow in 2006. This strain originated, along with the Aussie isolate of *A. areolatum*, in Sopron, Hungary. However, due to the loss of nematode infectivity following years of laboratory culture, the strain of nematode was reisolated from an early field site where it was released in Tasmania (Bedding 2009). Nematodes were kept under USDA, APHIS permit in a quarantine facility (the Sarkaria Arthropod Research Laboratory, Ithaca, New York) for the duration of the experiment. Nematodes were initially grown on *A. areolatum* Aussie using half-strength potato dextrose agar (Difco, Sparks, MD) with a total of 25 g/L agar to make this medium harder (1/2PDAh) (R.A. Bedding, pers. comm.). Petri dishes with nematodes were maintained inside brown paper bags in a 23^{o}C incubator with no light.

2.3.3 Fungal growth assay

The four isolates of *A. areolatum* were cultured on four types of media: green bean agar (GBA) (Dhingra and Sinclair 1995), Hagem agar (Hagem) (Stenlid 1985), potato dextrose agar plus 5 mg/L thiamine hydrochloride (PDAth) (Thomsen and Koch 1999), and 1/2PDAh. This latter medium is regularly used for maintaining this fungus commercially and therefore was considered a control. During experiments, cultures were maintained at 23^{o}C without lights.

For each fungal isolate, a plug was taken at the growing edge of a fungal colony, normally grown on 1/2PDAh, using a sterile 3 mm diameter cork borer and transferred to the center of a 100 mm diameter petri dish of the test media. In a few instances, to encourage the growth of enough fungus for the experiment, plugs were taken from PDAth. Fungal plugs were

allowed 4 days to begin growing on the new media before measurements began. Culture diameter was measured along two perpendicular axes every 48 h until either the fungal growth had filled the plate or had stopped growing for 2 measurement intervals (up to 44 d of observations). For each measurement interval, the area occupied by the fungal colony was calculated from the average of the two diameter measurements. This study was replicated 4-5 times with one dish per treatment for each replicate.

2.3.4 Nematode propagation assay

All nematode growth assays used the 1/2PDAh medium for growing *A. areolatum* and were conducted at 23ºC in darkness. The five isolates of *A. areolatum* were grown in 60 mm diameter Petri dishes by transferring a 3 mm diameter plug from the edge of a culture. The nematodes were then allowed to propagate for 1, 5, or 10 d at 23ºC in darkness, at which point each dish was inoculated with nematode eggs. To inoculate plates with eggs, *D. siricidicola* colonies were flooded with distilled water and the liquid containing nematodes and eggs was filtered three times through a Swinnex filter holder (Millipore) equipped with a 60 µm filter, which allowed eggs to pass through into the filtrate. In some instances, it was unavoidable that a few small juveniles passed through with the eggs, but this happened equally for all treatments. Ten 20 µL samples were taken from this stock suspension and all eggs were counted under a dissecting microscope at 20X magnification to volumetrically determine the total number of eggs in the stock solution. The solution was then diluted so that a mean of 167 eggs were present per 20 µL drop. Three drops were added to each fungal dish, equidistant from one another and half-way between the fungal plug and the edge of the Petri dish. Therefore, each dish received approximately 500 eggs. After 25 days, the dishes were flooded once with water and ten 20 µL samples of each of the washings were examined under a dissecting microscope to volumetrically

determine the total number of eggs and living nematodes per dish. The amount of water used for flooding differed by plate due to the varying amount of fungus and nematode density, but it was always measured for use in calculation of the total nematodes produced per plate. This study was conducted on two separate dates, with three replicates of each treatment on the first date and eight replicates of each treatment on the second date.

2.4 Data analysis

To calculate daily fungal growth, only data points from the log growth phase of fungal cultures were used. As a lower limit, we used the interval when the culture was > 8 mm in radius. Because cultures did not always grow to the Petri dish edges, we considered that fungal growth had stopped when the colony had grown 0.5 mm or less for two consecutive measurement intervals. Fungal growth rate (mm/d) was calculated by dividing the final diameter by the number of days of growth (during the aforementioned period). For analysis, fungal growth rates were log-transformed (log + 1) to evaluate the effects of media and isolate using a two-way general linear model (SAS 2002-2008). To compare individual isolates or medium, least mean square tests were conducted using the Bonferroni correction.

To compare the increase in nematode numbers when feeding on different fungal isolates, the numbers of nematodes (including both eggs and living nematodes) produced after 25 d were log-transformed. Fungal isolate and time of fungal growth before nematode inoculation were main effects using a multifactorial ANOVA and means were separated with LSMeans Tukeys HSD (JMP 2013). In a separate analysis, only the numbers of eggs produced after 25 d were log-transformed and analyzed using the same model as for total nematodes.

2.5 Results

2.5.1 Growth of fungal isolates

The interaction between media and fungal isolate was significant ($F_{12,53} = 2.82$; $P = 0.0046$) so separate analyses were conducted comparing growth of each isolate on different media. When comparing isolate growth on each type of media, the fungal isolate used for mass production of *D. siricidicola*, Aussie, grew faster than other isolates, on each of the media tested (Table 2.2). Rates of growth for the other three isolates did not differ significantly on GBA or Hagem agar and growth rates for isolates other than Aussie differed inconsistently on 1/2PDAh and PDAth (Table 2).

Each isolate varied in growth rate on different media. For each isolate, growth was consistently fast on green bean agar and slow on Hagem agar (Table 2.2). On Hagem agar, the commercial isolate Aussie grew to the edges of Petri dishes but other isolates stopped growing at 26.4-29.6 mm radius, which in all cases was significantly less growth than Aussie (Aussie versus Gr94-1: t = 7.4564, df = 1, $P < 0.0001$; Aussie versus ScyME: t = 5.8563, df = 1, $P < 0.0001$; Aussie versus SedDF: t = 7.7891, df = 1, $P < 0.0001$). During this study, we compared two adaptations of potato dextrose agar: 1/2PDAh versus PDAth. Growth rates on these media did not differ significantly for any isolate although for three of the four isolates, growth was numerically greater on PDAth than on 1/2PDAh. The fungal isolate from North American *S. nitidus* mycangia, ScyME, grew more slowly on all media compared with GBA (Table 2.2).

2.5.2 Nematode growth on fungal isolates

All *A. areolatum* isolates supported some growth of *D. siricidicola*. There was a significant interaction between the isolate and the time of fungal growth before nematode inoculation ($F_{8,152}$ = 2.31; P = 0.02), so subsequent analyses were performed to see which isolates in particular were significantly affected by days of fungal growth. Isolates for which days of growth was a significant factor were Aussie ($F_{2,31}$ = 3.45; P = 0.04), OtisAa ($F_{2,30}$ = 4.06; P = 0.03), and ScyME ($F_{2,26}$ = 12.99; P = 0.0001). In general, nematodes grown on OtisAa and ScyME produced more offspring as the number of days of fungal growth prior to inoculation increased (Fig. 2.1). The slowest growing isolate, ScyME, when given 10 d to grow prior to nematode inoculation, produced more nematodes ($4.7 \times 10^4 \pm 1.0 \times 10^4$; mean ± SE) than any other treatment given 10 d of fungal growth prior to inoculation with nematode (Fig. 2.2c). Isolates Gr94-1 ($F_{2,28}$=1.37; P = 0.27) and SedDF ($F_{2,31}$=1.35; P = 0.28) were not affected by days of fungal growth prior to inoculation. Isolate SedDF did not produce more nematodes than the initial inoculation in any treatment, despite nematodes developing to adults and reproducing (Fig. 2.1).

The total number of eggs produced had a significant interaction between fungal isolate x days of growth ($F_{8, 152}$ = 3.07; p = 0.003), so separate analyses were conducted. Nematodes produced eggs in all treatments (Fig. 2.3), although there was high variability between treatments. As with the total nematodes produced, ScyME produced numerically the most eggs with 10 d of fungal growth prior to inoculation compared with all other treatments ($3.3 \times 10^4 \pm 7.6 \times 10^3$). For three isolates (Aussie, OtisAa, and SedDF), the number of days of fungal growth prior to inoculation did not affect the number of eggs present at 25d (Fig. 2.3). Curiously, the number of eggs for Gr94-1 declined by the 10 d treatment while for ScyME the number of eggs

increased in the 10 d treatment. Numerically, the fewest eggs (27 $\pm$ 8) were produced when SedDF was grown for 10 d prior to inoculation with nematodes.

When all fungal isolates were given one day to grow prior to nematode inoculation, numbers of nematodes produced varied, although the fewest nematodes were produced on SedDF cultures (Fig. 2.2). Once the fungal isolates were given 5 or 10 d to grow, however, the numbers of nematodes in plates with Aussie, Gr94-1, and OtisAa were not significantly different from each other. In contrast, ScyME produced more nematodes when the fungus was given 10 d rather than 5 d to grow prior to inoculation with nematodes ($F_{2, 16} = 24.7$; $P = 0.0001$). Nematodes feeding on SedDF grew poorly in all treatments. However, it should be noted that although the number of nematodes grown on SedDF after 25 d did not exceed the initial inoculation of 500, nematodes feeding on this isolate laid eggs and the eggs hatched, so some degree of reproduction was possible. Experimental replicate had no significant effect ($F_{1,152} = 0.39$; $P = 0.5351$).

Table 2.2. Mean (± SE) millimeters per day radial fungal growth of four *Amylostereum areolatum* isolates growing on four different media.

	Amylostereum areolatum isolates			
Media[2]	Aussie	Gr94-1	ScyME	SedDF
Green bean agar (GBA)	4.7 ± 0.4 ab A	2.5 ± 0.2 a B	2.7 ± 0.3 a B	1.9 ± 0.1 a B
Hagem agar	3.3 ± 0.4 b A	0.9 ± 0.1 c B	1.0 ± 0.1 b B	0.9 ± 0.1 b B
½ strength potato dextrose agar, increased to 2.5% agar (1/2PDAh)	4.2 ± 0.1 ab A	1.1 ± 0.1 bc C	1.0 ± 0.1 b C	1.8 ± 0.1 a B
Potato dextrose agar + thiamine (PDAth)	4.9 ± 0.3 a A	1.9 ± 0.5 ab B	1.1 ± 0.1 b C	1.7 ± 0.1 a BC

[1] Different lower case letters by columns denote differences between mean fungal growth rates among types of media within isolate, and upper case letters denote differences across rows among isolates within media type (least square means using the Bonferroni correction to split the alpha = 0.05 across tests).

[2] Media described in the Materials and Methods.

Figure 2.1. Mean nematodes (+SE) produced per Petri dish after 25 d for five isolates of *Amylostereum areolatum* inoculated with nematodes after 1, 5, and 10 days of fungal growth, by isolate. Dashed line indicates the initial number of nematodes inoculated per dish. Different upper case letters denote differences between mean numbers of nematodes produced (separations given by LSMeans Tukey HSD test (JMP 2010)). Note differences in y axes.

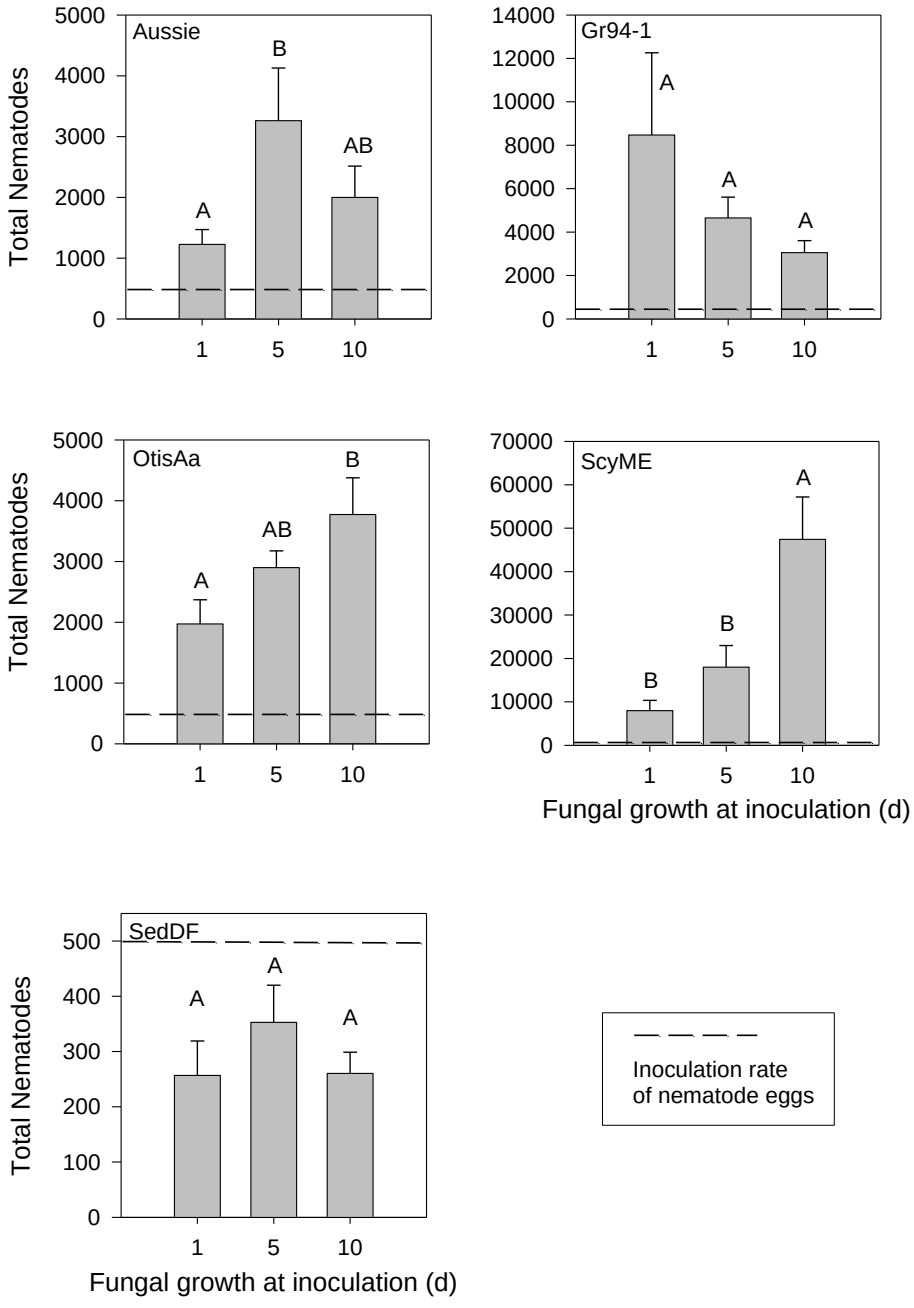


Figure 2.2. Mean nematodes (+SE) produced after 25 d for five isolates of *Amylostereum areolatum* inoculated with nematodes after 1, 5, and 10 days of fungal growth, by day. a. 1 day, b. 5 days and c. 10 days. Different upper case letters denote differences between mean numbers of nematodes produced (separations given by LSMeans Tukey HSD test (JMP 2010)).

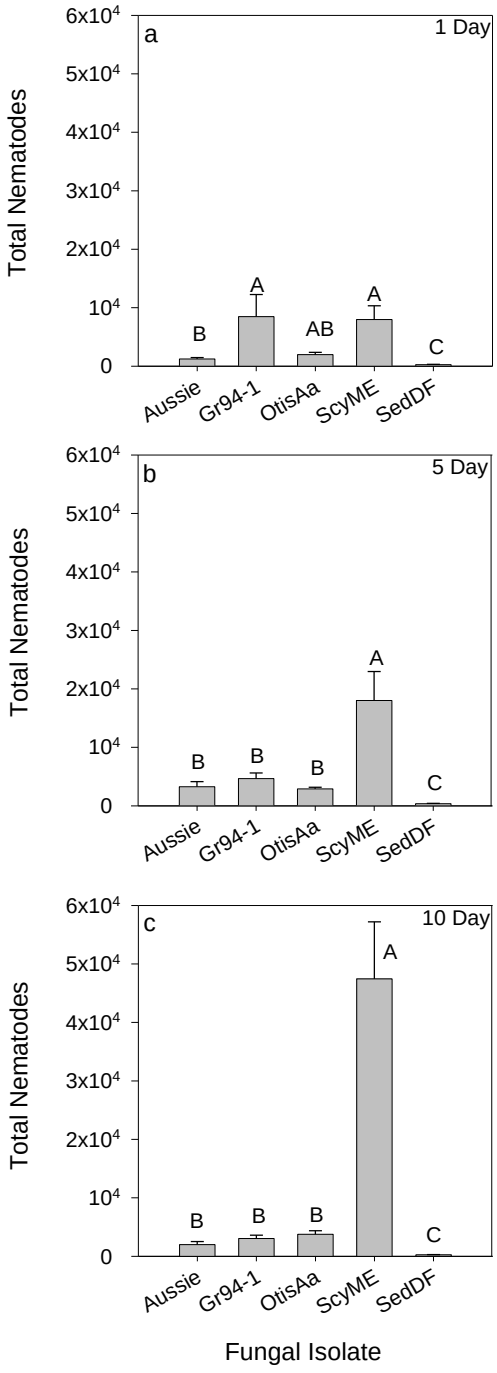
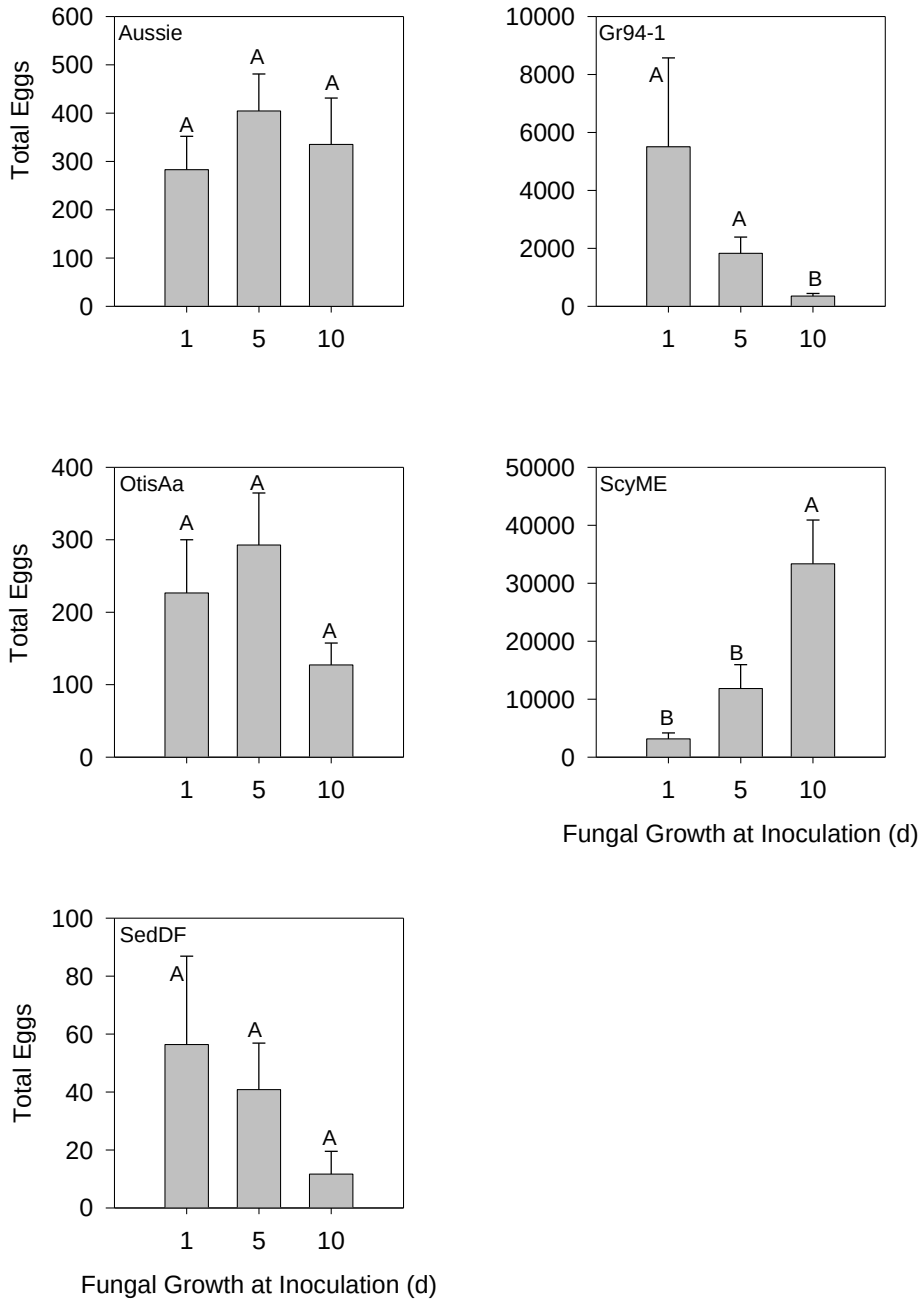


Figure 2.3. Mean nematode eggs (+SE) produced after 25 d for five isolates of *Amylostereum areolatum* inoculated with nematodes after 1, 5, and 10 days of fungal growth, by isolate. Different upper case letters denote differences between mean numbers of nematodes produced (separations given by LSMeans Tukey HSD test (JMP 2010)). Note differences in y axes.



2.6 Discussion

Deladenus siricidicola was able to persist when feeding on all *A. areolatum* isolates included in this study, despite the fact that growth rates for fungal isolates differed. However, nematode population numbers on the different fungal isolates varied enormously, ranging from very few nematodes produced (SedDF) to a nearly 100-fold increase in population (ScyME). Some fungal isolates established in North America supported more nematode propagation than the commercial fungal isolate. Interestingly, the fungal isolate that produced the most nematodes (ScyME) is native to North America.

Deladenus siricidicola only feeds on *A. areolatum* (Bedding and Akhurst 1978; A.E.H. & S.J.L. unpublished data) but *A. areolatum* occurs as different isolates. The isolates that *D. siricidicola* could potentially encounter in North America would depend on those isolates being carried by the resident *Sirex* species. *Sirex noctilio* alone carries at least two isolates of *A. areolatum* in North America (Nielsen et al. 2009; Bergeron et al. 2011). North American *Sirex* species were previously thought to carry only *Amylostereum chailletii* as their symbiotic fungus (Gilbertson 1984; Smith & Schiff 2002). However, the North American native species *Sirex* sp. '*nitidus*' has been collected carrying an isolate of *A. areolatum* that is presumed to be native to North America (IGS BE isolate) (Nielsen et al. 2009). When evaluating the potential for a biological control program using *D. siricidicola* in North America, the ability of the nematode to persist when feeding on native North American *A. areolatum* must be understood; if *D. siricidicola* is introduced into a tree where the native *A. areolatum* is established, survival and increase of the nematode would depend on its use of the native *A. areolatum* isolate. Our study demonstrates that the commercial *D. siricidicola* Kamona grows well when feeding on a native isolate of *A. areolatum* (ScyME), suggesting that presence of this fungal isolate in trees would

potentially promote growth of this biological control agent. In addition, *D. siricidicola* grew well on one IGS isolate of *A. areolatum* isolated from *S. noctilio* in North America (BD), although growth on the D isolate of IGS (SedDF versus OtisAa) carried by *S. noctilio* in North America was variable.

Cohabitation of the invasive and native *Sirex* species within trees, along with the ability of *D. siricidicola* to increase when feeding on different isolates of *A. areolatum*, could lead to close proximity of this biological control agent with native species of *Sirex*. Whether the commercial strain of *D. siricidicola* will parasitize the native *Sirex* is not known. Although the commercial strain of *D. siricidicola* has not been introduced into North America, a strain of *D. siricidicola* has been found in North America which parasitizes *S. noctilio* without causing female sterilization (Yu et al. 2009); an asynchrony between host and parasite physiology results in nematodes being present in the woodwasp ovaries but not within the woodwasp eggs (Zondag 1975; Yu et al. 2009; David W.Williams, unpubl. data). Thus, even if *D. siricidicola* parasitizes native North American *Sirex* woodwasps, it is not known if such an asynchrony between host and parasite physiology would occur in these hosts.

Contrary to our initial hypotheses, the two slowest growing fungal isolates produced the most nematodes. Bedding (1972) stated that because the mycophagous form of the nematodes only feed on the growing hyphal tips of a fungal colony, to grow *D. siricidicola* a balance must be achieved so that the *A. areolatum* culture does not overgrow the nematodes. In exploring the sides of the nematode/fungus "balance," our study failed to find a scenario in which the nematodes ate all of the fungal tips and then starved. However, nematode production on Aussie, the fastest growing isolate, may be evidence of the other side of the balance, where the fungus out-grew the nematodes, reaching the edge of the plate because nematode populations did not

increase fast enough to stop or slow the rapid fungal growth. A limitation of our study is that it took place in Petri dishes and therefore could not take into account interactions that may occur within fungal colonies in flasks used for commercial production of nematodes or when nematodes and fungus are growing within trees. Although *D. siricidicola* may be inoculated into a tree with the Aussie isolate of *A. areolatum*, Aussie would not necessarily become the prevalent fungus established in the tree. While Aussie has been inoculated into pine trees in South Africa along with *D. siricidicola*, it has not been recovered thus far in isolations from either the wood or from emerging *S. noctilio* (B. Slippers, pers. comm.). Additionally, it is not known how *D. siricidicola* and *Sirex* spp. will interact in trees hosting multiple isolates of *A. areolatum* or whether the nematodes would preferentially respond to different fungal isolates.

Vasiliauskas et al. (1998) used Hagem agar for growing *Amylostereum* species but for our studies, this medium consistently produced slow fungal growth. Our studies demonstrated that often fungal growth on GBA was faster than on other media, although differences were not always significant (Table 2.2). In studies in Denmark, *A. areolatum* was first grown on GBA to speed up growth before being moved to PDA, which produced slower growth (Thomsen & Koch 1999). While we did not test full strength PDA for comparison, the addition of thiamine to full strength PDA did not significantly increase growth when compared with 1/2PDAh. Thomsen and Koch (1999) reported that the addition of thiamine to PDA resulted in growth equivalent to GBA, but in our study this was only true for three of four isolates; the fungal isolate ScyME grew faster on GBA than on PDA plus thiamine.

The ability of *D. siricidicola* to differentially survive and propagate on different isolates of *A. areolatum* could affect numerous aspects of a biological control program for *S. noctilio* using *D. siricidicola*. First, fungal isolates could be chosen to optimize mass production of *D.*

siricidicola, based on the ability of the nematodes to increase in population numbers on a given isolate. Second, if *D. siricidicola* in a tree encounters alternate isolates of *A. areolatum* that have already colonized parts of that tree, the ability of the nematodes to persist on the established isolate of *A. areolatum* could lead to success or failure of *S. noctilio* within that tree. Lastly, the same ability of *D. siricidicola* to grow and increase when feeding on a given fungal isolate of *A. areolatum* could lead to non-target effects if *D. siricidicola* has the ability to parasitize and is in proximity with the native siricids associated with *A. areolatum* in the same tree.

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Chapter 3

Deladenus (Tylenchida: Neotylenchidae) feeding on species and isolates of the white rot fungus *Amylostereum* in pines[2]

3.1 Abstract

Deladenus siricidicola nematodes are used for the biological control of invasive *Sirex noctilio* woodwasps in the Southern Hemisphere. Since the discovery of established *S. noctilio* in North America and Canada in 2005, a similar biological control program is under consideration in the United States. In this study, a culture of *Deladenus* nematodes was established from a native *Sirex nigricornis* woodwasp collected in New York State. These nematodes were identified as *Deladenus proximus* using molecular and morphometric techniques. *D. siricidicola* 'Kamona' are mass produced for biological control when feeding on the white rot fungus *Amylostereum areolatum* in the Southern Hemisphere, so we compared the relative development and reproduction of mycophagous forms of both nematode species when feeding on native and invasive isolates of *Amylostereum* fungus. *D. siricidicola* Kamona were able to reproduce on all isolates of *A. areolatum* tested, but reproduced poorly on the *A. areolatum* isolate they would be most likely to encounter in northeastern North America, should the nematode be released. *D. proximus* were able to reproduce well on both *A. chailletii* and *A. areolatum*, despite prior

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evidence suggesting only *A. chailletii* is a suitable food source. This is the first report of the ability of *D. proximus* to survive and reproduce on *A. areolatum*, the fungal symbiont usually carried by *Sirex noctilio*, suggesting this native nematode should be evaluated for its ability to parasitize and sterilize *S. noctilio*.

Keywords: *Deladenus*, *Amylostereum*, *Sirex noctilio*, biological control, mycophagy, diet breadth

3.2. Introduction

Deladenus siricidicola Bedding (Tylenchida: Neotylenchidae) was the first nematode to be used commercially for pest control, and its use to control the invasive pine-killing woodwasp, *Sirex noctilio* F. (Hymenoptera: Siricidae), is one of the most successful classical biocontrol projects of its kind (Bedding and Iede, 2005). *D. siricidicola*, as well as some other nematodes of the genus *Deladenus*, live a remarkable dual lifestyle which alternates between fungus-feeding and insect-parasitic (Bedding, 2009). The fungal-feeding form (= mycophagous) lives inside trees while feeding on white rot fungi of the genus *Amylostereum* (Fries) Boidin (Basidiomycota: Russulales). The parasitic form of *D. siricidicola* infects woodwasp larvae of the genus *Sirex*. Parasitized adult female *Sirex* emerging from a tree act to disperse the nematodes when they oviposit in a new tree. *Sirex* woodwasp larvae are able to develop in trees due to *Amylostereum*, a common resource of both the nematode and the woodwasp. Hyphal fragments, or oidia, of *Amylostereum* spp. are carried in specialized internal organs called mycangia within adult female *Sirex*, and oidia are injected into the tree at oviposition. Thus, *Sirex* woodwasps disperse both the nematodes and the food source for mycophagous *Deladenus*. Because parasitism often results in

sterilization of the female woodwasp, *Deladenus siricidicola* has been used extensively for biological control of *S. noctilio* in the Southern Hemisphere (Hurley et al., 2007).

Sirex noctilio is native to Eurasia and northern Africa, where it is not considered a pest. However, where it has been introduced in the Southern Hemisphere, it has caused extensive damage to plantations of introduced pines (Hurley et al., 2007). *S. noctilio* successfully infest pine trees (*Pinus* spp.) by injecting oidia of *A. areolatum* along with phytotoxic venom during oviposition. The fungus and venom inhibit the tree's ability to defend against the fungal invasion. *S. noctilio* larvae survive and grow only in the presence of *A. areolatum* (Madden and Coutts, 1979). Since being identified in the northeastern United States in 2005, *S. noctilio* has spread throughout New York and into Pennsylvania by 2006, to Vermont and Michigan by 2007, to Ohio by 2009, to Connecticut by 2010, and to New Jersey by 2012 (NAPIS, 2013).

Deladenus nematodes can produce many generations in the mycophagous phase, and this is exploited in the Southern Hemisphere in order to mass-produce *D. siricidicola* Kamona for biological control. This nematode owes its success as a biological control agent of *S. noctilio* in the Southern Hemisphere, in part, to the introduced nature of pine plantations there. In the absence of native pines, non-target effects are drastically reduced because there are no native *Sirex* species to parasitize and no native *Deladenus* species with which to compete, although several hymenopteran parasitoids have been introduced in addition to nematodes. However, pines are native to North America and North American forests are home to multiple species of *Sirex* woodwasps (Schiff et al., 2012). Some native *Sirex* species carry *Amylostereum chailletii* F. (Boidin) as their fungal symbiont, although *Sirex nigricornis* F. and *Sirex nitidus* (Harris) are now known to carry *A. areolatum* naturally at least sometimes (Hajek et al., 2013). The isolates of *A. areolatum* found in native *Sirex* spp. are different from those found in *S. noctilio* based on

intergenic spacer regions (IGS), and are assumed to be native to North America (Nielsen et al., 2009; Hajek et al., 2013).

Additionally, native *Deladenus* nematode species parasitize North American *Sirex* (Chapter 4). In particular, the pine-dwelling native *Sirex nigricornis* is parasitized by *Deladenus proximus* (Bedding 1974; Yu et al., 2011). There is potential for interaction between fungal and nematode communities associated with *S. noctilio* and *S. nigricornis*, due to their mutual affinity for pine trees. These *Sirex* species have been found infesting the same trees, and *S. nigricornis* has been found to carry an isolate of *A. areolatum* known to have originated from *S. noctilio* (Nielsen et al., 2009; Hajek et al., 2013). In addition to fungal exchange, two instances of nematode exchange have been recorded. Two *S. nigricornis* specimens collected in New York State were parasitized by a strain of non-sterilizing *D. siricidicola* that was introduced to North America with *S. noctilio* and one *S. noctilio* in New York State was found carrying *D. proximus*, which is normally associated with *S. nigricornis* (Chapter 4).

D. proximus was originally described from parasitized *S. nigricornis* in South Carolina, USA in 1974 (Bedding and Akhurst 1978). Recently, Yu et al. (2011) found nematodes parasitizing *S. nigricornis* in Ontario, Canada, and identified them as *D. proximus* via morphological and molecular methods. The nematodes are difficult to identify in that they must first be reared to adulthood in culture before morphology can be used; additionally, there has been a scarcity of molecular work on known *Deladenus* species with which to compare sequences from unidentified *Deladenus*. *D. proximus* has not previously been recorded from the state of New York, so to confirm the identification of nematodes parasitizing *S. nigricornis* included in this study, nematodes were reared to adults and identified using both morphological and molecular methods.

Whereas *D. siricidicola* has been studied extensively due to its status as a biological control agent, relatively little is known about the biology of *D. proximus*. When Bedding and Akhurst (1978) described *D. proximus* in *S. nigricornis* in the southeastern U.S., they found that the mycophagous form of the nematode was only able to eat *A. chailletii,* and not *A. areolatum.* Thus, it was eliminated as a potential biological control agent of *S. noctilio* (Bedding and Iede 2005). Similarly Yu et al (2011) found that *D. proximus* parasitizing *S. nigricornis* in Ontario, Canada, only matured to the adult stage when feeding on *A. chailletii* isolates. With the lone exception of *Deladenus wilsoni* Bedding, most insect-parasitic *Deladenus* reproduce when feeding on either *A. areolatum* or *A. chailletii* but not both (Bedding and Akhurst, 1978). However, Chapter 4 describes multiple *S. nigricornis* parasitized by *D. proximus* while simultaneously carrying *A. areolatum* in their mycangia, indicating the possibility that the *D. proximus* parasitizing those *S. nigricornis* developed in association with *A. areolatum.*

To explore possible new associations between the fungus and nematode communities associated with *S. noctilio* and *S. nigricornis* in North America, we were interested in the ability of *D. siricidicola* and *D. proximus* to feed on species and isolates of *Amylostereum* currently present in North America. We hypothesized that *D. proximus* would be able to reproduce on the native North American isolate of *A. areolatum,* in addition to *A. chailletii*. Several isolates of *A. areolatum,* including the isolate used for mass production of *D. siricidicola* in Australia, were established and subsequently inoculated with *D. siricidicola* or *D. proximus* eggs in order to investigate the relative increase of the nematode species on the different isolates of fungus.

3.3 Materials and Methods

3.3.1 *D. siricidicola* culture

The strain of *D. siricidicola* used for studies, now commonly called Kamona, was obtained in 2006 from Ecogrow Environment (Queanbeyan, N.S.W., Australia) where it is mass-produced for biological control. This strain originated in Sopron, Hungary around 1967. However, after the loss of nematode infectivity following years of laboratory culture was discovered in the 1980s, the strain of nematode was reisolated from an early field site where it had been released in Kamona, Tasmania (Bedding, 2009).

D. siricidicola Kamona were kept under USDA, APHIS permit in a quarantine facility (the Sarkaria Arthropod Research Laboratory, Ithaca, New York) for the duration of the experiment. These nematodes were initially grown on 100 mm diameter Petri dishes containing cultures of the isolate of *A. areolatum* used in the commercial production of *D. siricidicola*, IGS isolate BDF, using half-strength potato dextrose agar (Difco, Sparks, MD) with a total of 25 g/L agar to make this medium harder (1/2PDAh) (R.A. Bedding, pers. comm.). Petri dishes with fungus and nematodes were maintained inside brown paper bags in a 23^{o}C incubator with no lights.

3.3.2 Isolation of *D. proximus*

Sirex nigricornis specimens were obtained by felling red pine (*Pinus resinosa*) and Scots pine (*Pinus sylvestris*) trees exhibiting symptoms of *S. noctilio* infestation described by Hoebeke et al.

(2005). Felled trees were cut into bolts approximately one meter in length and ends were waxed to prevent the wood from drying quickly. Bolts were put into fiber barrels (77.5 cm tall x 51.4 cm diam.) with window screening covering the top and kept at ambient conditions in a barn. During *Sirex* emergence periods, barrels were checked several times each week. *S. nigricornis* males were kept alive at 4°C in 29.6mL clear plastic cups with lids until dissection could confirm the presence of juvenile nematodes in the testes. A culture of *D. proximus* was established by inoculating the juvenile nematodes from a parasitized *S. nigricornis* male specimen that emerged from red pine (*Pinus resinosa)* from Warren County, New York on September 7, 2012, onto 100 mm diameter Petri dishes containing cultures of *A. areolatum* BE isolate, using 1/2PDAh medium. Nematodes were maintained as a mycophagous culture inside brown paper bags in a 23ºC incubator with no light.

3.3.3 Fungal isolates

The geographic and host origins of the seven species and isolates of *Amylostereum* included in this study are listed in Table 3.1. Two isolates of *A. chailletii* were included in the study. Four of the *A. areolatum* isolates were collected in North America and chosen to represent the diversity of genotypes present in North America based on intergenic spacer regions (IGS) (Nielsen et al., 2009; Hajek et al., 2013). Four of the isolates used in this study are thought to be native to North America (WB9/19, SniDF, ScyME, and LLASni11/20-10) and two isolates are thought to have been introduced along with *S. noctilio* (Gr94-1, and SedDF).

Table 3.1. Isolates of *Amylostereum* included in the reproduction assays.

SAC #	Isolate ID	Species	Isolation from	Original host	Date Isolated	IGS Isolate
132	WB9/19[1]	*A. areolatum*	Warrensburg, NY	*S. nigricornis*	19 Sep 2009	BE
081	ScyME[2]	*A. areolatum*	Waldo Co., ME	*S. nitidus*	13 Sep 2007	BE
101	Gr94-1[1,2]	*A. areolatum*	Fulton County, NY	*S. noctilio*	19 Feb 2008	BD
085	SedDF[1,2]	*A. areolatum*	Oswego County, NY	*S. nigricornis*	19 Sep 2007	D
001	Aussie[1]	*A. areolatum*	Sopron, Hungary	*S. juvencus*	1967	BDF
091	SniDF[2]	*A. chailletii*	New Haven, NY	*S. nigricornis*	21 Sep 2007	G
152	LLASni-11/20-10[2]	*A. chailletii*	Kisatchie NF, LA	*S. nigricornis*	20 Nov 2008	G

[1]Isolate used for *D. siricidicola* Kamona reproduction assay.

[2]Isolate used for *D. proximus* reproduction assay.

3.3.4 *S. nigricornis* nematode identification

3.3.4.1 Molecular characterization

Nematode colonies obtained by rearing nematodes from *S. nigricornis* were identified using molecular characterization via amplification and sequencing of the mtCO1 gene. The primers used, PCR conditions, and sequencing methods were described in Chapter 1. The sequence was then compared to *D. proximus* mtCO1 sequences in GenBank (X10427).

3.3.4.2 *D. proximus* morphometrics

Adult mycophagous *D. proximus* were preserved according to Bedding (pers. comm.) as follows: Nematodes were washed from a Petri plate and the suspension, containing approximately 500 nematodes in 1mL tap water, was placed into a 15mL centrifuge tube. The tube was put into a 60deg C water bath for 1min to kill the nematodes. The tube was next filled with 10mL TAF fixative (2% triethanolamine and 8% formalin). Nematodes were allowed to settle, at which point excess TAF was removed. Adult nematodes were placed on slides, with five per slide. Two pieces of vellum paper were placed under either side of each 22x22mm cover slip to prevent the weight of the cover slip from crushing the nematodes. Slides were then placed on a slide warmer (Chicago Surgical Electrical Co., Melrose Park, IL). The TAF was replaced slowly with a 50:50 ethyl alcohol (ETOH):glycerol solution by adding 10µl of the solution to the edge of the cover slip and allowing it to diffuse. This was done to prevent the high density glycerol from crushing or distorting the nematodes. Slides were then left on the slide warmer at 50^{o} C for seven days, adding ETOH:glycerol daily as the TAF and ETOH evaporated. Twenty each of adult male and

female *D. proximus* were measured and morphometrics were recorded using characters described by De Man (1880) (Table 3.2). Nematodes were examined and under a Leica DM2500 microscope with DIC capabilities and an attached DFC295 camera (Leica Microsystems, Wetzlar, Germany). Measurements were taken in Leica Application Suite 3.7 (LASv3.7) and compared to measurements of *D. proximus* provided by Yu et al. (2011) and Bedding (1974).

3.3.5 Nematode propagation assay

The goal of these studies was to compare *D. siricidicola* or *D. proximus* increase when feeding on native *A. areolatum* (IGS BE), introduced *A. areolatum* (IGS BD, D), or the commercial isolate (IGS BDF). All nematode growth assays used 1/2PDAh medium for growing *Amylostereum* isolates and were conducted at 23^{o}C in darkness. The fungal isolates were grown in 100 mm diameter Petri dishes by transferring a 3 mm diameter plug from the growing edge of a culture. The fungal cultures were grown for 5d, at which point each dish was stored at 4^{o}C until it was inoculated with nematode eggs. To inoculate plates with nematode eggs, *D. siricidicola* Kamona and *D. proximus* colonies were flooded with sterile distilled water containing 6.25mg/L gentocin. The liquid containing nematodes and eggs and fungus was filtered three times through a Swinnex filter holder (Millipore) equipped with a 60 µm filter, which allowed eggs to pass through into the filtrate. In some instances, it was unavoidable that a few small juveniles passed through with the eggs, but this happened equally for all treatments. Ten 20 µL samples were taken from this stock suspension and all eggs were counted under a dissecting microscope at 20X magnification to determine the total number of eggs in the stock solution volumetrically. The suspension was then diluted so that a mean of 167 eggs were present per 20 µL drop. Three

drops were added to each fungal dish, equidistant from one another and half-way between the fungal plug and the edge of the Petri dish. Therefore, each dish received approximately 500 eggs. Inoculated Petri dishes were placed in paper bags and allowed to propagate for up to 34d.

Starting at 7d following nematode inoculation, plates were destructively sampled every 3d for the remainder of the experiment. For each collection date, two to four plates per treatment were flooded with water. The amount of water used for flooding differed by plate due to the varying fungal and nematode densities, but it was always measured for calculation of the total nematodes produced per plate. Ten 20 µL samples of each of the washings were examined under a dissecting microscope at 20X magnification to volumetrically determine the total number of eggs and nematodes per dish. For the *D. siricidicola* study, the number of available nematode eggs for inoculating plates was a limiting factor, so fewer replicate plates were set up (from two to four), and the experiment was conducted on five separate dates for four isolates of *A. areolatum*. *A. chailletii* was not tested due to the inability of *D. siricidicola* to grow on *A. chailletii* (Bedding and Akhurst 1978). For the *D. proximus* experiment, there were a total of 30 dishes of nematodes per each of the five fungal treatments (3 *A. areolatum*, 2 *A. chailletii*) for a total of 150 plates per experiment, and the experiment was conducted on three separate dates. *D. proximus* was not tested due to the authors' expectation that *A. areolatum* BDF will not be released in the U.S.

3.4 Statistical analysis

To compare the increase in nematode numbers when feeding on different fungal isolates, the total numbers of either *D. siricidicola* or *D. proximus* (including eggs, juveniles and adults)

produced over the course of the experiments were transformed to log (x + 1). Fungal isolate and days post nematode inoculation were the main effects using a multifactorial ANOVA with experimental replicate coded as a random effect, and means were separated with least squared means Tukey's HSD (JMP 2010). To determine differences in production of either *D. siricidicola* or *D. proximus* among fungal isolates for specific times, separate analyses were conducted, in which day was the main effect in one-way ANOVAs. The total number of nematodes produced per fungal isolate was compared and means were separated with least squared means Tukey's HSD (JMP 2010). To determine differences in total egg production of either *D. siricidicola* or *D. proximus* among isolates, the mean numbers of eggs for each treatment were calculated and then transformed to log (x + 1). Fungal isolate was the main effect in a one-way ANOVA with experimental replicate coded as a random effect, and means were separated with LSMeans Tukey's HSD (JMP 2010).

3.5 Results

3.5.1 *S. nigricornis* nematode identification

The twenty reared nematodes of each sex that were evaluated matched the original description of *D. proximus* (Bedding, 1974) as well as recent data from *D. proximus* parasitizing *S. nigricornis* in Canada (Yu et al., 2011). The sample size (n = 20 of each sex) was greater than the original description (n = 12 of each sex) and greater than the Yu et al. (2011) study (n = 11 females, n = 7 males). The results from the morphometric analysis of the nematodes included in the study fit within the previously defined parameters for *D. proximus* (Table 3.3).

The mtCO1 sequence for the *S. nigricornis* nematode used in the experiment was identical to previously identified *D. proximus* included in Chapter 4. The sequence was submitted to Genbank (BankIt1623083).

Table 3.2. Morphometric features used to differentiate species in this study.

Morphometric Characters	Symbol[1]
Body length	L
Body length/maximum body width	A
Body length/esophageal length	B
Body length/tail length	C
(Distance from cloacal aperture to anterior end of testes/body length)*100	T
% distance of vulva from anterior	V
(Distance from cloacal aperture to anterior end of ovaries/body length)*100	G_1
Excretory pore to anterior end	
Excretory pore anterior to hemizonid	
Tail length	

[1]Characters are referred to by these symbols in Table 3.3.

Table 3.3. Morphometric measurements of mycophagous adults of *D. proximus*. Range is given in parentheses in the row under the mean ± SE.

Characters[1]	Yu et al., 2011 Measurements		Bedding, 1974 Measurements		*Deladenus* from *S. nigricornis* Measurements	
	Female	Male	Female	Male	Female	Male
# Specimens	11	7	12	12	20	20
Body length(mm) (L)	2.38 ± 0.21	1.39 ± 0.18	2.03	1.52	2.04	1.43
	(1.93 - 2.91)	(1.02 - 1.59)	(1.76-2.2)	(1.34-1.59)	(1.46-2.49)	(1.19-1.68)
Body width (µm)	49.12 ± 11.23	16.81 ± 1.73	-	-	45.48	19.99
	(32.55 - 71.19)	(13.83 - 19.02)	-	-	(33.55-56.49)	(17.80-23.77)
Stylet length (µm)	10.06 ± 0.67	10.06 ± 0.61	11.4	11	11.18	10.85
	(8.07 - 10.13)	(8.82 - 10.05)	(11-12)	(11)	(9.98-12.7)	(9.46-11.77)
Excretory pore from the anterior end (µm)	145.11 ± 25.73	95.33 ± 12.76	127	126	149.08	-
	(134.23 - 150.89)	(89.99 - 111.96)	(127-159)	(111-142)	(142.98-155.17)	-
Excretory pore anterior to the hemizonid (µm)	7.23 ± 4.41	7.33 ± 1.19	8.52	2.1	8.01	-
	(2.21 - 11.14)	(4.57 - 8.80)	(1-11)	(0-7)	(6.46-9.55)	-
Tail length (µm)	33.93 ± 3.96	40.02 ± 5.03	39	48	35.86	41.33
	(30.24 - 44.68)	(27.94 - 45.15)	(31-47)	(44-52)	(30.41-43.38)	(34.66-46.10)

A	50.19 ± 6.47	81.47 ± 7.82	47.6	56	45.20	71.66
	(35.50 - 50.33)	(65.00 - 91.87)	(40-53.7)	(48-62)	(38.00-58.41)	(55.58-83.46)
B	16.78 ± 3.33	18.56 ± 4.66	19.1	15.5	-	-
	(13.13 - 19.90)	(12.56 - 22.91)	(16.3-21.3)	(13.6-17.5)	-	-
C	65.19 ± 6.39	34.30 ± 3.19	53	32	55.89	34.61
	(55.15 - 75.53)	(28.84 - 38.00)	(44-60)	(27-35)	(47.49-64.53)	(28.3-40.74)
V	96.14 ± 0.86	-	90.3	-	95.58	-
	(95.69 - 97.11)	-	(87.3-93.9)	-	(92.32-96.49)	-
G_1	83.23 ± 6.91	-	88.9		-	-
	(75.55 - 89.12)	-	(84.8-94.8)		-	-
T	-	78.59 ± 6.45	-	90.3	-	86.67
	-	(71.00 - 86.45)	-	(87.3-93.9)	-	(81.98-90.45)

[1]Symbols are defined in Table 3.2.

3.5.2 Nematode growth assay

3.5.2.1 D. siricidicola Kamona feeding on isolates of A. areolatum

D. siricidicola Kamona consistently attained a population density above the initial inoculation number per plate of 500 nematodes on all fungal isolates except *A. areolatum* IGS D (Fig. 3.1). There was an interaction between isolate and day ($F_{3, 404}$=31.04; p <0.0001). Separate analyses on isolate and day showed that *A. areolatum* BE and *A. areolatum* BD produced significantly more nematodes than *A. areolatum* BDF and *A. areolatum* D over time ($t_{1,103}$= 3.79; p < 0.0001) (Fig. 3.2). *A. areolatum* BE and *A. areolatum* BD produced the most nematodes over all days combined, with averages of $2.5 \times 10^4 \pm 3.78 \times 10^3$ (mean + SE) and $9.40 \times 10^3 \pm 9.63 \times 10^2$, respectively ($F_3$, 404 = 40.1; p < 0.0001). *A. areolatum* isolates BDF and D produced the fewest nematodes, with $9.10 \times 10^2 \pm 1.53 \times 10^2$, and $4.17 \times 10^2 \pm 34$, respectively.

Nematodes on *A. areolatum* isolates BE and BD exceeded 500 by days 10 or 13, and nematodes on *A. areolatum* isolate D never exceeded 500 (Fig. 3.1). *A. areolatum* BE and *A. areolatum* BD also produced the most eggs over all days combined, with averages of 7.9×10^3 and 1.9×10^3, respectively ($F_{3, 41}$ = 26.0; p < 0.0001) (Fig. 3.3).

3.5.2.2 Growth of D. proximus on isolates of A. areolatum and isolates of A. chailletii

D. proximus consistently maintained a population well above the initial inoculation number of 500 nematodes when feeding on *A. areolatum* BE and the New York isolate of *A. chailletii* (Fig. 3.1). There was an interaction between isolate and day ($F_{4,438}$ = 7.14; p < 0.001). Separate analyses on isolate and day showed that *A. chailletii* New York, *A. areolatum* BE, and *A. areolatum* BD produced significantly more nematodes than *A. chailletii* Louisiana and *A.*

areolatum D over time ($t_{1, 88} = 2.79$; $p = 0.007$) (Fig. 2.4). *A. chailletii* New York and *A. areolatum* BE produced the most nematodes over all days combined, with averages of $4.84 \times 10^3 \pm 8.60 \times 10^2$ and $3.48 \times 10^3 \pm 5.73 \times 10^2$, respectively ($F_{4,443} = 89.86$; $p < 0.001$) (Fig. 3.1). Limited reproduction occurred by *D. proximus* on *A. areolatum* D and the Louisiana isolate of *A. chailletii*, with average numbers of nematodes of $1.13 \times 10^2 \pm 9$ and $2.04 \times 10^2 \pm 40$, respectively.

Nematodes were able to lay eggs on all isolates except *A. areolatum* isolate D, on which nematodes never reproduced. The time at which eggs laid exceeded 500 was 10-13d for the New York isolate of *A. chailletii*, and ranged from 10-16d for plates of *A. areolatum* isolate BE (Fig. 3.4). In only one experimental replicate of three did *D. proximus* growing on *A. areolatum* isolate BD exceed 500, and this occurred between 16-19d. *A. chailletii* New York and *A. areolatum* BE also produced the most eggs over all days combined, with averages of 7.9×10^3 and 1.9×10^3, respectively ($F_{3, 38} = 50.1$; $p < 0.0001$) (Fig. 3.3).

Figure 3.1. Total combined numbers (±SE) *D. siricidicola* (A) and *D. proximus* (B) nematodes and eggs produced on fungal isolates. Capital letters denote significant differences between total nematodes and eggs produced on a given fungal isolate. Horizontal line indicates initial number of eggs inoculated.

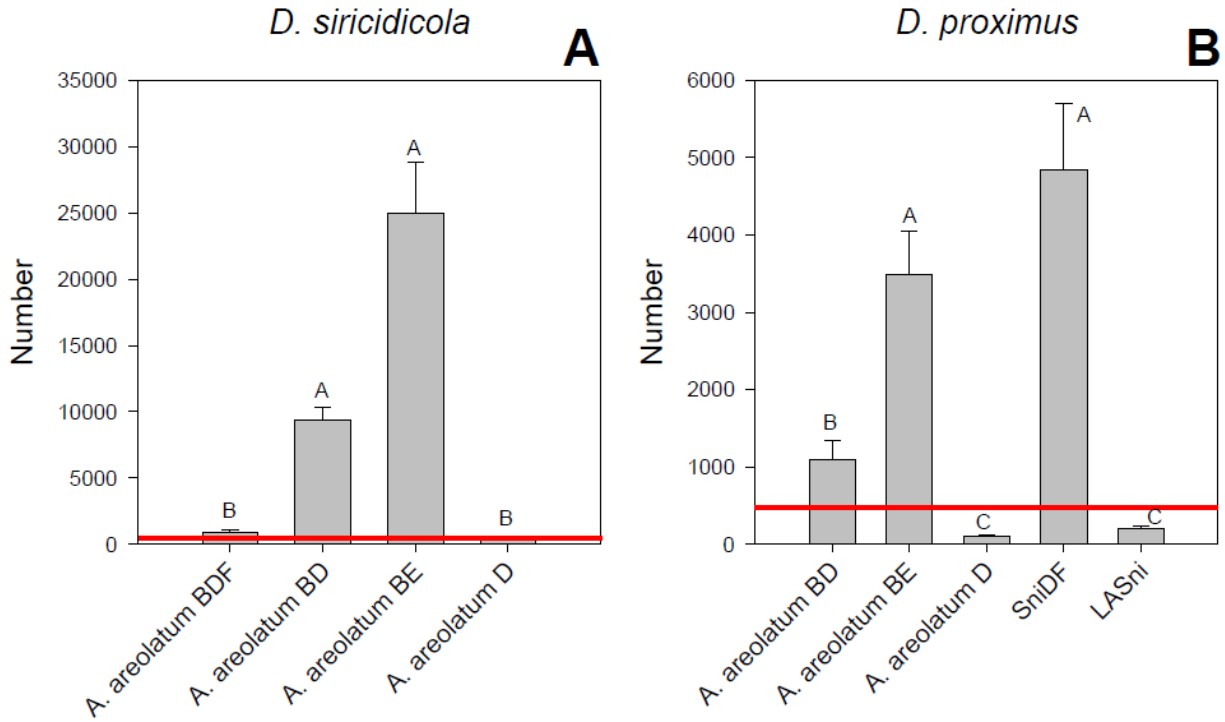


Figure 3.2. Mean numbers of *D. siricidicola* nematodes and eggs produced on four isolates of *A. areolatum* over 34d. Upper case letters denote significant differences in the total of nematodes and eggs produced on different fungal treatments over all days combined. Horizontal line indicates initial number of eggs inoculated

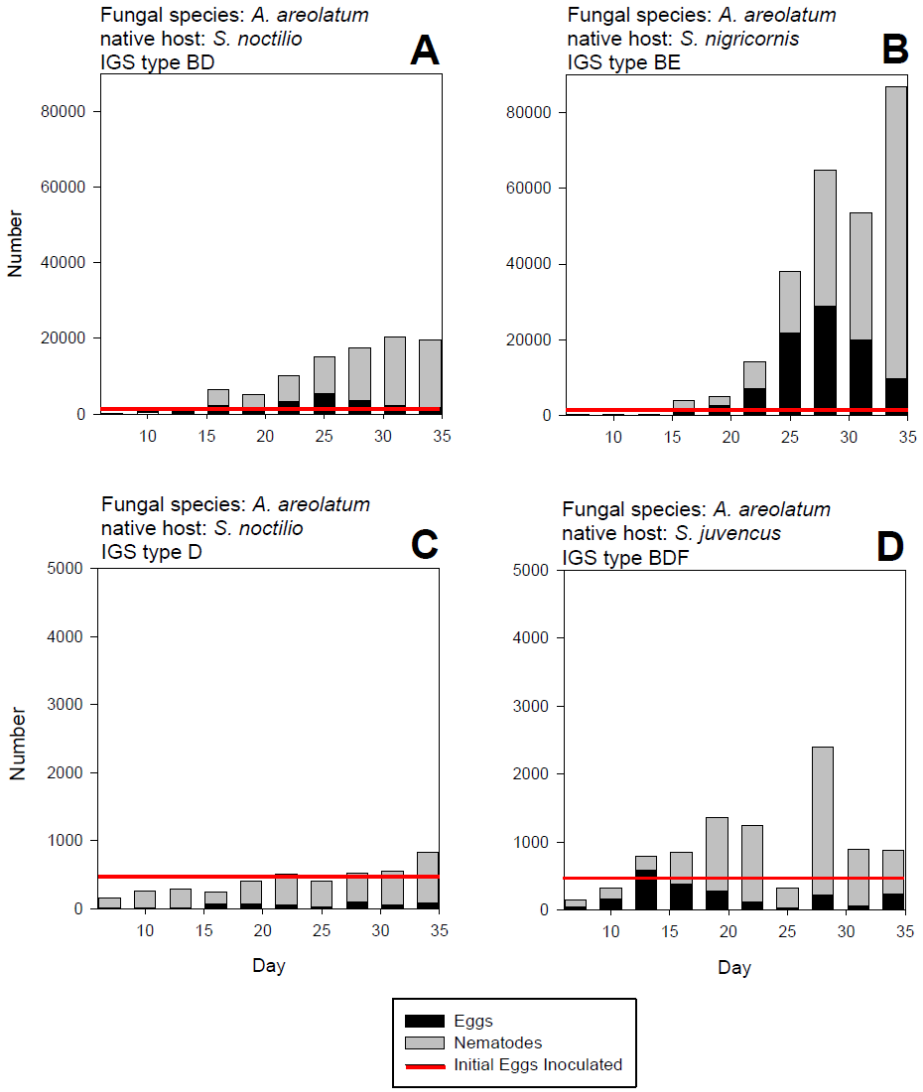


Figure 3.3. Mean numbers (±SE) of *D. siricidicola* and *D. proximus* eggs produced on fungal isolates. Capital letters denote significant differences between eggs produced among fungal isolates.

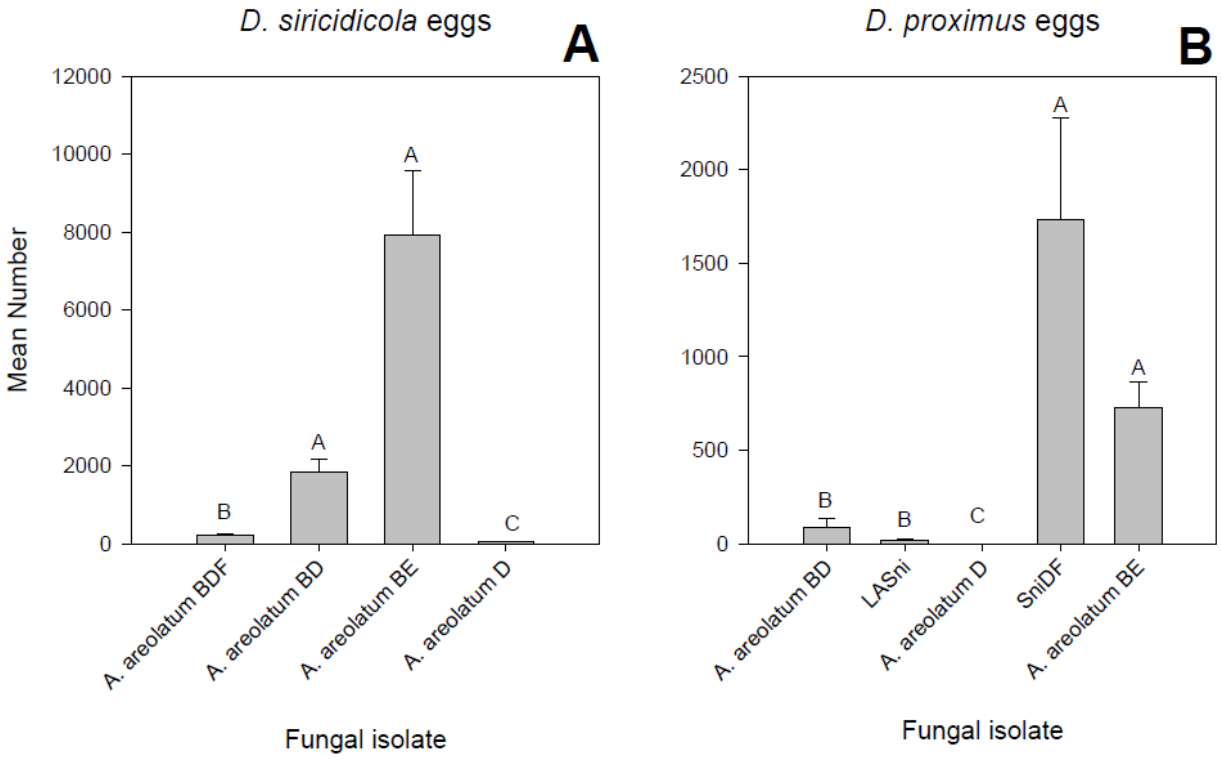
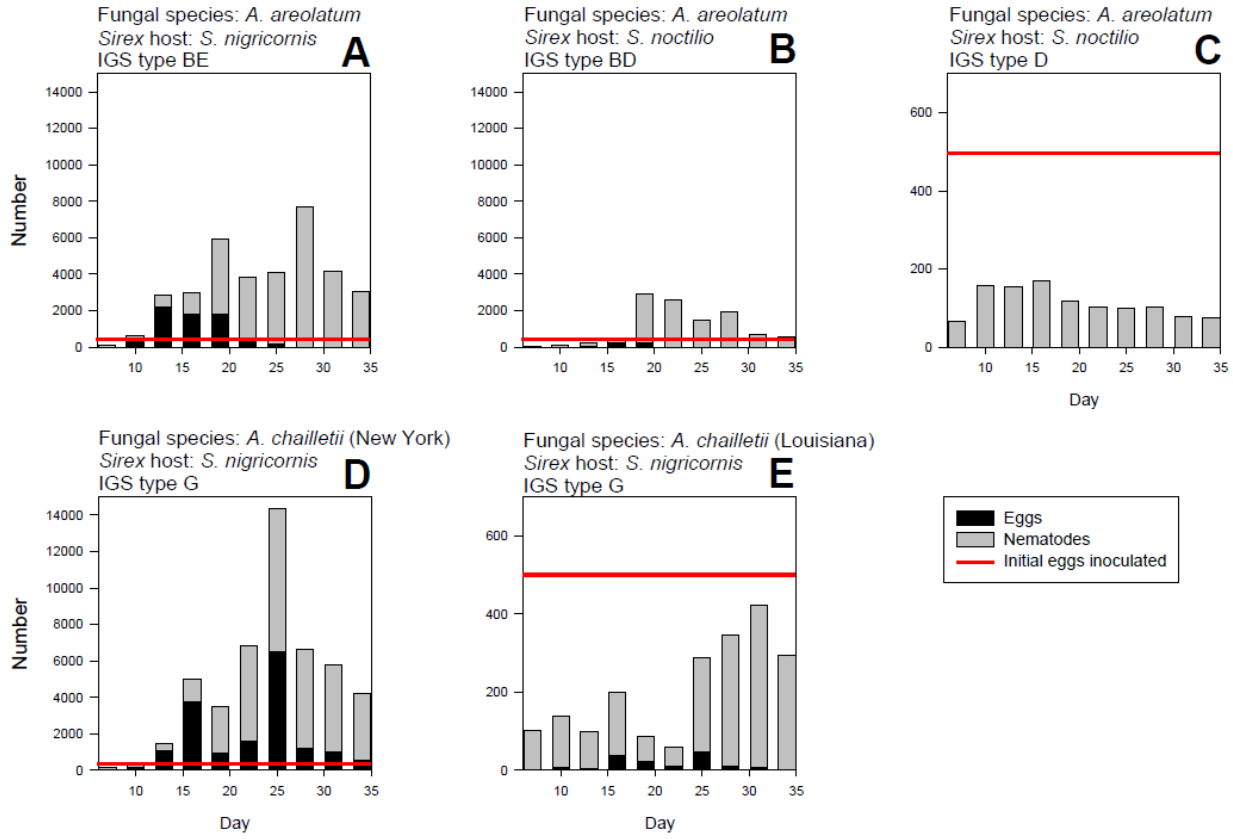


Figure 3.4. Mean numbers (±SE) of *D. proximus* nematodes and eggs produced every 3d on three isolates of *A. areolatum* and two isolates of *A. chailletii* over 34d. Horizontal line indicates initial number of eggs inoculated.



3.6 Discussion

Deladenus nematodes are introduced into a tree as the mycophagous form, whether via parasitized *S. noctilio* or manually as part of a biological control program. This makes the type of fungus available in the tree extremely important to the growth, survival, and reproduction of the nematodes, which ultimately will affect the ability of the nematodes to form infectives and parasitize new *S. noctilio* larvae. Previous studies found that *D. proximus* exclusively feeds on *A. chailletii*; Bedding and Akhurst (1978) and more recently Yu et al. (2011) found that *D. proximus* juveniles could only be reared to adults on isolates of *A. chailletii*. Based on this finding, *D. proximus* was not evaluated as a possible biological control agent of *S. noctilio* (Bedding and Iede 2005). In this study, however, *D. proximus* developed into adults and reproduced on two of the three *A. areolatum* isolates tested, in addition to *A. chailletii*. Attention has been called towards the possible need to find additional strains of *Deladenus siricidicola* for biological control of *S. noctilio*, because it is hypothesized that *S. noctilio* had multiple founding populations, making the introduction of a single strain of nematode for biological control insufficient to control the genetically heterogeneous mixture of *S. noctilio* (Mlonyeni et al., 2011). Based on this need for nematode diversity, *D. proximus* should be evaluated for its ability to parasitize and sterilize *S. noctilio* in North America.

D. proximus has been found in New York State parasitizing *S. nigricornis* that carried the *A. areolatum* BE isolate in their mycangia, leading to the speculation that this fungal isolate would be a suitable host food for the nematode (Chapter 4). In this study, the two fungal isolates which produced the most *D. proximus* were *A. chailletii* (New York) and *A. areolatum* BE isolate. These two isolates are notable in that both are thought to be native to North America and were therefore fungal symbionts of *S. nigricornis* prior to the *S. noctilio* invasion (Nielsen et al., 2009;

Hajek et al., 2013; Wooding et al., 2013). A prior history between *D. proximus* and *A. areolatum* BE isolate may be the reason that the nematode can thrive on *A. areolatum* BE isolate but not on *A. areolatum* BD and D isolates, which are both thought to have been introduced to North America with the *S. noctilio* invasion.

Chitambar (1991) reported that insect-parasitic *Deladenus* nematodes only could be separated via morphometric measurements into one of two supergroups--the *D. wilsoni* supergroup, including *D. wilsoni* and *D. proximus*, and the *D. siricidicola* supergroup, containing all of the other insect-parasitic *Deladenus*. Since then, Yu et al. (2011) separated *D. wilsoni* and *D. proximus* based on distances between the anterior of the nematode, the excretory pore, and the hemizonid. However, given that *D. wilsoni* can grow on both species of *Amylostereum* (Bedding and Akhurst 1978), it is reasonable that *D. proximus*, which is hypothesized to be closely related to *D. wilsoni*, also is able to grow on both fungal species. Notably, *D. wilsoni* historically was considered for the control of *S. noctilio*, but was eliminated due to its propensity to parasitize hymenopteran parasitoids of *S. noctilio* (Bedding and Iede, 2005).

Interestingly, the isolate of *A. areolatum* on which *D. proximus* reproduced well, *A. areolatum* BE isolate, is also the isolate on which *D. siricidicola* Kamona produced numerically the greatest number of offspring, despite the apparent lack of evolutionary history of *D. siricidicola* with this fungal isolate, which has not yet been found in Europe (Nielsen et al., 2009; Hajek et al., 2013; Wooding et al., 2013). Likewise, both species of nematode reproduced poorly on *A. areolatum* isolate D, which originated from invading *S. noctilio*. *D. siricidicola* Kamona reproduced very little on this isolate, despite developing into adults, while *D. proximus* never matured into adults, and thus did not reproduce at all when feeding on it.

This association between *Deladenus siricidicola* Kamona and *A. areolatum* D isolate could have unintended consequences in a biological control program. This fungal symbiont is widespread within *S. noctilio* populations in North America. Hajek et al. (2013) found that 25 out of 27 *S. noctilio* tested carried this isolate as their fungal symbiont, based on IGS sequence data. *A. areolatum* symbionts of *S. noctilio* also have been commonly found with this IGS type in North America in other studies (Bergeron et al., 2011; Wooding et al., 2013). Given the poor reproduction of *D. siricidicola* Kamona on this particular fungal isolate, adequate biological control may be difficult to achieve, if target *S. noctilio* are surrounded by a protective layer of non-host fungus, upon which the nematode will not develop properly.

To avoid releasing another non-native *A. areolatum* isolate in North America during biological control introductions, it was suggested in Chapter 2 that *A. areolatum* BE isolate be considered for mass production of *D. siricidicola* Kamona, should this nematode be released to control *S. noctilio*. At that time, however, it was not known that *D. proximus* and *D. siricidicola* could cross-infect their respective hosts (Chapter 3), nor was it known that *D. proximus* could thrive when feeding on certain isolates of *A. areolatum*.

A. areolatum BE isolate, which has not been found in *S. noctilio*, is vegetatively incompatible with *A. areolatum* BD isolate, which is carried by *S. noctilio* (Hajek et al., 2013). This suggests the possibility that using *A. areolatum* BE isolate to mass produce *D. siricidicola* Kamona for biocontrol could lead to a vegetative incompatibility barrier in a tree between the injected nematodes and target *S. noctilio* larvae. Would *D. siricidicola* Kamona cross the barrier to parasitize the larvae? Or, consider another scenario involving injecting *D. siricidicola* Kamona and *A. areolatum* BE isolate into a pine tree in which *D. proximus* are present. If the *D. proximus* benefitted from a population boost due to the presence of more of their fungal food

source, they might then outcompete *D. siricidicola* Kamona and parasitize the invasive *S. noctilio*. The presence of a *Deladenus* nematode does not necessarily lead to sterilization of a given *Sirex* host. Indeed, *S. noctilio* invading North America brought with them a different strain of *D. siricidicola*. This strain, called the non-sterilizing strain, invades the *S. noctilio* reproductive system; however, juvenile nematodes remain external to the eggs, leading to viable *S. noctilio* offspring (Yu et al., 2009; Williams et al., 2012). Even in the absence of sterilization, however, the non-sterilizing strain of *D. siricidicola* can have physiological impacts on the host, including reduced body size and lowered fecundity (Kroll et al., 2013). It is presently unknown whether *D. proximus* parasitizing *S. noctilio* would be sterilizing or non-sterilizing.

Fungal host can influence reproduction of mycophagous nematodes (Townshend, 1964). In the present study, *D. siricidicola* and *D. proximus* did not reproduce equally on different fungal treatments. This may be attributed to multiple factors ranging from poor nutrition (Pillai and Taylor 1976) to nematicidal toxins (Jansson and Lopez-Llorca, 2001) of a given fungal isolate.

In another scenario, eggs and nematodes instead could be overgrown and parasitized by fungus. An advantage to the destructive sampling design of this study was the ability to observe the numbers of eggs produced in a given treatment, and when those eggs appeared. A prior study showed that *D. siricidicola* Kamona reproduced poorly on *A. areolatum* IGS D, and this study investigated this further (Chapter 2). We found that low yields of nematodes in different fungal treatments could not necessarily be attributed to the same cause. For example, although numbers of *D. proximus* on *A. chailletii* (Louisiana) and *A. areolatum* D isolate were statistically the same, there were observable differences. On *A. chailletii* (Louisiana), nematode eggs present at inoculation were frequently overgrown by fungus and subsequently never hatched, but the few surviving juveniles were sometimes able to develop into adults and reproduce (EEM unpublished

data). On the contrary, most *D. proximus* eggs on *A. areolatum* D hatched but the juveniles did not reach the adult stage and therefore did not reproduce.

Other lignicolous fungi are fed upon by mycophagous nematodes (Townshend, 1964) and fungi have evolved different mechanisms of dealing with this grazing pressure. Barron (1977) suggested that wood-rotting fungi in particular could benefit from an antagonistic relationship with nematodes. First, nitrogen is generally limited in wood, so preying on nematodes could provide a nutritional boost for wood-rotting fungi. Second, when nematodes are fungivorous, there would be an added benefit for these fungi if there are fewer nematodes to graze upon fungal hyphae. In this study, when nematodes were able to lay large numbers of eggs, isolates of both species of fungus were often observed growing over eggs en masse (EEM, unpublished data); after nematode eggs were overgrown by *Amylostereum*, they became shrunken and never hatched, suggesting that the fungus is killing the nematode eggs. Whether this naturally occurs within trees is not known, nor is it known how the fungal colony is ultimately affected by using the nematode eggs for nutrition or by reducing the overall number of nematodes.

Tanney and Hutchison (2011) described a novel nematode antifeedant mechanism employed by the basidiomycete *Sphaerobolus* spp., in which the stylets of *Aphelenchoides* nematodes pierced special fungal structures called gloeocystidia, resulting in the anterior portion of the nematodes becoming swollen and covered with an encapsulating matrix. This matrix prevented further nematode feeding, leading to death of the immobilized nematodes, possibly due to starvation. *Amylostereum* fungi have a different type of cystidia called "encrusted cystidia," which are thought to have a function in secretion (Gull and Newsam 1975). Tanney and Hutchison (2011) discuss the role that cystidia play in fungal defense, and it is possible that *Amylostereum* cystidia play a similar role against fungal grazers.

Nutrition also could play a role in the ability of *Deladenus* to successfully reproduce when feeding on *Amylostereum* species and isolates. Pillai and Taylor (1968) tested the influence of fungal species on the population increase of multiple species of fungivorous nematodes and attributed lower populations of nematodes to an absence in some fungi of an essential nutrient required for nematode reproduction. The authors also successfully linked nematode morphometric measurements to fungal food source. Nutritional differences between species and isolates of *Amylostereum* have not been described or quantified, though they certainly could exist.

In conclusion, this study provides evidence that species and isolates of the symbiotic *Amylostereum* fungus carried by *Sirex* woodwasps impact reproduction of *Deladenus* nematodes. *D. proximus* can thrive on native North American *A. areolatum* BE isolate, despite prior evidence suggesting it only eats *A. chailletii*. *D. siricidicola* Kamona, the biological control agent of *S. noctilio* in the Southern Hemisphere, is able to thrive on multiple isolates of *A. areolatum*, including an isolate obtained from *S. nigricornis* which historically had no association with *D. siricidicola* Kamona.

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Chapter 4

Phylogenetic Analysis of *Deladenus* Nematodes Parasitizing Northeastern North American *Sirex* Species[3]

4.1 Abstract

The parasitic nematode *Deladenus siricidicola* is a biological control agent of the invasive woodwasp, *Sirex noctilio*. Since the discovery of *S. noctilio* in pine forests of northeastern North America in 2005, a biological control program involving the Kamona strain of *D. siricidicola* has been under consideration. However, North American pine forests have indigenous *Sirex* spp. and likely harbor a unique assemblage of associated nematodes. We assessed phylogenetic relationships among native *Deladenus* spp. in the northeastern United States and the Kamona strain of *D. siricidicola.* We sequenced three genes (mtCO1, LSU, and ITS) from nematodes extracted from parasitized *Sirex* spp. collected inside and outside of the range of *S. noctilio*. Our analyses suggest cospeciation between four North American *Sirex* spp. and their associated nematode parasites. Within two *S. noctilio* individuals we found nematodes that we hypothesize are normally associated with *S. nigricornis*. One individual of the native *Sirex nigricornis* contained *Deladenus* normally associated with *S. noctilio*. We discuss nematode-host fidelity in

[3] Morris, E.E., [a] Kepler, R.M., [a] Long, S.J., [a] Williams, D.W., [b] Hajek, A.E., [a] 2013. Phylogenetic analysis of *Deladenus* nematodes parasitizing northeastern North American *Sirex* species. J. Invertebr. Pathol. 113, 177-183.

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this system and the potential for non-target impacts of a biological control program using *D. siricidicola* against *S. noctilio*.

Key Words: Host specificity; biological control; *Amylostereum*; woodwasp; invasive species; forest entomology

4.2 Introduction

Deladenus species are parasitic nematodes that attack siricid woodwasps and also some siricid parasitoids and associates (Bedding and Akhurst 1978) that develop in conifers (Williams et al. 2012). Some species in the genus *Deladenus* (=*Beddingia*) have a dicyclic life history, consisting of a free-living mycophagous life cycle and an insect-parasitic life cycle (Chitambar 2001). These different strategies allow *Deladenus* to increase within trees as the specific wood rot fungus they eat grows, then switch to the parasitic form when siricid hosts are present, facilitating their dispersal to new trees.

In the eastern United States, there are three native species of *Sirex*: *S. nigricornis, S. cyaneus,* and *S. nitidus* (Schiff et al. 2012). Additionally one introduced species, *S. noctilio*, was first collected in New York state in 2004 (Hoebeke et al. 2005). *Sirex* species infest trees by depositing eggs along with a symbiotic white rot fungus into the tree during oviposition. The symbiotic fungi, in the genus *Amylostereum*, grow throughout the wood and provide nutrition to the *Sirex* larvae. North American *Sirex* are not considered serious pests as they only infest dead or dying trees (Furniss and Carolin 1977; Madden 1988); however, the invasive *S. noctilio* is capable of killing healthy trees (Spradbery 1973).

Most of what is known about *Deladenus* comes from in-depth studies of its use as a biological control agent against *S. noctilio* (Bedding and Iede 2005). One part of the *D. siricidicola* life cycle is spent living within the tracheids of pine trees. There, nematodes eat the growing hyphal tips of the white rot fungus *A. areolatum* (Basidiomycota: Russulales), the fungal symbiont of *S. noctilio*. Adult mycophagous nematodes mate via amphimixis, lay eggs, and develop from larvae into adults. This free-living cycle can continue indefinitely. In the presence of *S. noctilio* larvae, however, chemical cues stimulate nematode larvae to develop into preinfective adults. Preinfective adults also mate via amphimixis and the mated females use a long tubular stylet to pierce the *Sirex* larval cuticle and enter the host. Once inside, the nematode becomes parasitic after it sheds its outer cuticle and develops microvilli on the outside of its body, facilitating absorption of nutrients from the host. This leads to rapid growth of the nematode. When the host pupates, the nematode lays eggs that will develop into mycophagous adults (Bedding 2009). The eggs hatch and juvenile nematodes migrate to the host's reproductive organs. Depending on the species and strain of *Deladenus*, juveniles can be found either within host egg shells, or around viable host eggs. In parasitized male *S. noctilio*, juvenile nematodes migrate to the testes, but a male *S. noctilio* host is a dead end for the nematode. When a parasitized female emerges from the tree as an adult, it mates and then oviposits on a new tree, injecting nematodes that are either within or around the eggs (Bedding 2009).

Deladenus siricidicola juveniles migrate into *S. noctilio* eggs, effectively sterilizing the host. This sterilizing effect the nematode has on the host has been exploited in order to reduce populations of *S. noctilio* in the Southern Hemisphere in numerous biological control programs (Hurley et al. 2007). Upon the arrival of *S. noctilio* to North America, controlled release studies were conducted on the use of the *D. siricidicola* to control *S. noctilio* in the United States

(Williams et al. 2012). The use of *D. siricidicola* in the United States may be more complex than in the Southern Hemisphere, however, in that unlike the Southern Hemisphere, where pines are introduced and there are no native *Sirex*, North America has native pines, indigenous *Sirex*, and associated nematodes.

During a worldwide survey conducted in the 1970s to find natural enemies of *Sirex noctilio*, seven species of *Deladenus* parasitizing *Sirex* species and their associates were described from North America (Bedding and Akhurst 1978; Bedding 1974). However, the distribution of these nematodes was not well defined as samples were only collected from a few areas (See Table 4.1). Further complicating matters, a non-sterilizing strain of *D. siricidicola* has been found parasitizing *S. noctilio* in Ontario, Canada and in New York state (Shields 2009; Williams et al. 2009), which presumably arrived with this invasive (Yu et al., 2009). This strain is referred to as the "North American strain" by Williams et al. (2012) and the "non-sterilizing form" by Yu et al. (2009). The relationships among species within *Deladenus* are poorly understood and no phylogenetic analyses focused solely on the group have been conducted. The seven North American insect-parasitic species of *Deladenus* were described based on the morphology of mycophagous adults (Bedding 1968, 1974). However, in a review of this entire genus, Chitambar (1991) stated that morphology cannot be used to distinguish species of *Deladenus* that parasitize *Sirex* except for defining two major groups, or superspecies. The two proposed superspecies are *Deladenus wilsoni* and *Deladenus siricidicola*.

In this paper, we use molecular methods to characterize the diversity and relationships of *Deladenus* species associated with both native and introduced *Sirex* woodwasps in the eastern United States. Understanding the diversity of these nematodes and their host associations may

inform decisions about the impacts of nematodes introduced for *S. noctilio* biocontrol in this region.

4.3 Materials and Methods

4.3.1 Specimen acquisition and DNA extraction

Sirex woodwasps were obtained from multiple sites in New York, Pennsylvania, and Louisiana from 2008-2011 through a combination of intercept-panel traps (www.alphascents.com), insect collecting nets, and rearing from infested wood. Intercept-panel traps were placed 1 – 2 m high in pine trees and checked one to four times monthly. After collection, *Sirex* woodwasps were kept at 4°C in 29.6mL plastic cups with lids until dissection. The majority of *Sirex* specimens were obtained by felling red pine (*Pinus resinosa*) and Scots pine (*Pinus sylvestris*) trees exhibiting symptoms of *Sirex* infestation described by Haugen and Hoebeke (2005) and cutting the felled trees into bolts approximately one meter in length. The bolts were placed in barrels with a screened lid and kept in a lab at room temperature or at ambient conditions in a barn. During *Sirex* emergence periods, barrels were checked several times each week. All *Sirex* were kept alive at 4°C until dissection could confirm the presence of nematodes. We obtained a live culture of *D. siricidicola* Kamona (specimen "noc172"), a biological control agent of *S. noctilio* that is mass produced by the company Ecogrow Environment (Queanbeyan, N.S.W., Australia). Another live culture, of the non-sterilizing North American strain of *D. siricidicola* (specimen "noc173") was isolated from *S. noctilio* from Manlius, New York. Specimen data for nematodes and *Sirex* hosts are given in Table 4.2

Table 4.1. Species of *Deladenus* found in eastern United States and Canada.

Nematode	Insect host	Tree host	Fungal food source	Collection location	Citation
D. canii	*S. cyaneus*	*Abies balsamea*	*Amylostereum chailletii*	New Brunswick, Canada	Bedding (1974)
D. proximus	*S. nigricornis*	*Pinus* spp.	*Amylostereum chailletii*	South Carolina, United States	Bedding (1974)
D. wilsoni	*S. cyaneus*, *Rhyssa* spp.	(any tree with rhyssines or *S. cyaneus*)	*Amylostereum chailletii*, *Amylostereum areolatum*	United States, Canada, wherever rhyssines parasitizing *Sirex* occur	Bedding (1968)
D. siricidicola (North American strain)	*S. noctilio*	*Pinus* spp.	*Amylostereum areolatum*	New York, United States, and Canada	Yu et al. 2009

Table 4.2. *Sirex* nematodes and outgroup samples used for DNA sequencing

						GenBank Accession No.		
Host	Host source	Coll. Date	Coll. Location	Sirex sex	Nematode ID#	MTCO1	LSU	ITS
S. nigricornis	Trap	9-Sep-09	Oswego, NY	f	nig4	JX104234	JX104233	
S. nigricornis	Trap	5-Oct-10	Warrensburg, NY	f	nig159	JX104269		
S. nigricornis	Trap	5-Oct-10	Warrensburg, NY	f	nig161	JX104270	JX104269	JX212772
S. nigricornis	Trap	5-Oct-10	Warrensburg, NY	f	nig162	JX104271		JX212773
S. nigricornis	*Pinus taeda*	1-Nov-10	Grants Parrish, LA	f	nig163	JX104272	JX104271	JX212774
S. nigricornis	*Pinus taeda*	1-Nov-10	Grants Parrish, LA	f	nig164	JX104273		JX212775
S. nigricornis	*Pinus taeda*	1-Nov-10	Grants Parrish, LA	f	nig165	JX104274	JX104273	
S. nigricornis	Trap	15-Oct-08	Mt. Morris, PA	f	nig12	JX104240	JX104239	JX212752
S. nigricornis	Trap	19-Oct-08	Garards Fort, PA	f	nig14	JX104242	JX104241	JX212754
S. nigricornis	caught in field	18-Sep-10	Warrensburg, NY	f	nig157	JX104268	JX104267	JX212771
S. nigricornis	*Pinus resinosa*	2008	Fabius, NY	m	nig175	JX104278	JX104277	JX212779
S. nigricornis	*Pinus resinosa*	2008	Fabius, NY	m	nig174	JX104277		JX212778
S. nigricornis	Trap	14-Oct-08	Mount Morris, PA	f	nig13	JX104241		JX212753
S. nigricornis	*Pinus resinosa*	2008	Fabius, NY	m	nig176	JX104279		JX212780
S. nitidus	*Picea abies*	2-Sep-09	Newcomb, NY	m	nit30	JX104245		JX212756
S. nitidus	*Picea abies*	28-Aug-08	Newcomb, NY	f	nit17	JX104243		JX212755
S. cyaneus	*Abies balsamea*	31-Aug-09	Newcomb, NY	f	cya2	JX104232		JX212748
S. cyaneus	*Abies balsamea*	31-Aug-09	Newcomb, NY	f	cya3	JX104233		
S. cyaneus	*Abies balsamea*	31-Aug-09	Newcomb, NY	m	cya5	JX104235	JX104234	
S. cyaneus	*Abies balsamea*	31-Aug-09	Newcomb, NY	m	cya6	JX104236	JX104235	
S. cyaneus	*Abies balsamea*	29-Aug-09	Newcomb, NY	m	cya7	JX104237	JX104236	JX212749
S. cyaneus	*Abies balsamea*	29-Aug-09	Newcomb, NY	m	cya8	JX104238	JX104237	JX212750
S. cyaneus	*Abies balsamea*	25-Aug-09	Newcomb, NY	m	cya9	JX104239	JX104238	JX212751
S. cyaneus	*Abies balsamea*	29-Aug-09	Newcomb, NY	f	cya24	JX104244	JX104243	
S. cyaneus	*Abies balsamea*	31-Aug-09	Newcomb, NY	m	cya32	JX104246	JX104245	
S. cyaneus	*Abies balsamea*	14-Aug-09	Newcomb, NY	m	cya34	JX104247		JX212757
S. cyaneus	*Abies balsamea*	21-Aug-09	Newcomb, NY	m	cya36	JX104248	JX104247	JX212758
S. cyaneus	*Abies balsamea*	21-Aug-09	Newcomb, NY	m	cya37	JX104249		JX212759
S. cyaneus	*Abies balsamea*	26-Aug-09	Newcomb, NY	m	cya38	JX104250	JX104249	
S. cyaneus	*Abies balsamea*	17-Aug-09	Newcomb, NY	m	cya41	JX104251		
S. cyaneus	*Abies balsamea*	21-Aug-09	Newcomb, NY	m	cya43	JX104252	JX104251	

S. cyaneus	*Abies balsamea*	25-Aug-09	Newcomb, NY	m	cya50	JX104253		
S. cyaneus	*Abies balsamea*	26-Aug-09	Newcomb, NY	f	cya52	JX104254	JX104253	JX212760
S. noctilio	*Pinus sylvestris*	22-Jun-09	Oswego, NY	m	noc76	JX104255		JX212761
S. noctilio	*Pinus sylvestris*	25-Jun-09	Oswego, NY	m	noc78	JX104256	JX104255	
S. noctilio	*Pinus sylvestris*	30-Jun-09	Oswego, NY	m	noc79	JX104257		JX212762
S. noctilio	*Pinus sylvestris*	22-Jun-09	Oswego, NY	m	noc80	JX104258	JX104257	JX212763
S. noctilio	*Pinus resinosa*	10-Jul-09	Tioga, PA	m	noc101	JX104259		JX212764
S. noctilio	*Pinus sylvestris*	2-Jul-09	Onondaga, NY	f	noc115	JX104260	JX104259	
S. noctilio	*Pinus sylvestris*	1-Jul-09	Onondaga, NY	m	noc119	JX104261		JX212765
S. noctilio	*Pinus sylvestris*	6-Jul-09	Onondaga, NY	m	noc120	JX104262	JX104261	JX212766
S. noctilio	*Pinus sylvestris*	25-Jun-09	Onondaga, NY	m	noc121	JX104263		JX212767
S. noctilio	*Pinus sylvestris*	19-May-09	Onondaga, NY	m	noc124	JX104264	JX104263	JX212768
S. noctilio	*Pinus sylvestris*	26-May-09	Oswego, NY	m	noc148	JX104265		JX212769
S. noctilio	*Pinus sylvestris*	26-May-09	Oswego, NY	m	noc149	JX104284	JX104283	JX212770
S. noctilio	Trap	3-Sep-10	Warrensburg, NY	f	noc155	JX104266	JX104265	
S. noctilio	*Pinus resinosa*	2011	Huron, NY	m	noc180	JX104281		JX212782
S. noctilio	Net	27-Aug-10	Warrensburg, NY	f	noc156	JX104267		
S. noctilio	*Pinus resinosa*	2011	Huron, NY	m	noc179	JX104280	JX104279	JX212781
S. noctilio	*Pinus resinosa*	11-Jul-11	Triangle, NY	m	noc192	JX104282	JX104281	
S. noctilio	*Pinus resinosa*	2011	Huron, NY	m	noc193	JX104283		
S. noctilio	NA		NA		noc172[b]	JX104275		JX212776
S. noctilio	*Pinus sylvestris*		Manlius, NY	f	noc173[c]	JX104276	JX104275	JX212777
S. noctilio			Australia		AY633450[d]	AY633450		
S. noctilio			Ontario, CA		EU545474[e]	EU545474		
S. noctilio			Ontario, CA		FJ004889[e]			FJ004889
S. nigricornis			Ontario, CA		JF304744[f]			JF304744
Drosophila neotestacea			Rochester, NY		how367[g]	AY589466	AY589395	

[a]*Sirex*-Nematode collection numbers, Cornell University, Ithaca, NY

[b]Live culture mass produced by the company Ecogrow Environment (Queanbeyan, N.S.W., Australia) (=Kamona strain).

[c]Live culture obtained from D. W. Williams at the USDA, APHIS Otis Laboratory in Buzzards Bay, MA.

[d]*D. siricidicola*, sequence data from Ye et al. (2007).

[d]*D. siricidicola*, sequence data from Yu et al. (2009).

[f]*D. proximus*, sequence data from Yu et al. (2011).

[g]Outgroup. *Howardula aoronymphium*, sequence data from Ye et al. (2007).

Nematodes were preserved in 95% ethanol until subsequent tissue lysis and DNA extraction. DNA was extracted using a QIAamp DNA Micro Kit (Qiagen, Valencia, CA) after removing nematodes from the ethanol and lysing them by soaking in a waterbath at 56°C overnight. For female *Sirex*, approximately 4 eggs showing symptoms of nematode infection either inside or on the outside of the eggs were used in the extraction process. Nematode DNA was obtained from male *Sirex* specimens by extraction from testes. Two samples were obtained from live cultures of nematodes, and in this case, the nematode colony was flooded with 95% ethanol and approximately 10µL of the suspension was included in the DNA extraction for all samples. DNA was eluted in double distilled H_2O and stored at -20°C until use as a PCR template.

4.3.2 DNA amplification, sequencing, and analysis

Primers used for PCR amplification and sequencing are listed in Table 4.3. Reaction conditions for amplification of mtCO1 and LSU were the same as those in Ye et al. (2007). For the ITS gene, the thermal cycling program was the same as that used by Subbotin et al. (2001 & 2006).

PCR products were purified for sequencing using a QIAQuick PCR Purification Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions and eluted in double distilled H_2O. PCR products were sequenced in both directions by the Core Laboratory Center (CLC) at Cornell University. Raw sequence data were assembled and edited with CodonCode Aligner (version 3.7.1). The sequence data for the outgroup included in the study was obtained from Genbank. The outgroup was the nematode *Howardula aoronymphium* (Tylenchida: Allantonematidae), which is in the same suborder as *Deladenus* (Hexatylina) and is parasitic on

mycophagous *Drosophila* (Ye et al. 2007). Sequence alignment for each gene was performed in MAFFT (Katoh et al. 2002; Katoh and Toh 2008) and improved by direct examination in Mesquite (version 2.74). Gaps were treated as missing data. jModelTest (version 0.1.1) (Posada 2008; Guindon and Gascuel 2003) was used to select the most appropriate model of nucleotide substitution for each gene under the AIC criterion. Tree configurations resulting from maximum likelihood (ML) analyses performed in RAxML with 100 bootstrap replicates for individual gene datasets did not reveal any conflict among mtCO1 and ITS genes, so a combined dataset was created for mtCO1 and ITS. Significant tree differences were observed in the LSU dataset (ML bp>70), and this gene was analyzed separately. For all ML analyses in RAxML, the GTRgamma model was used, as the model suggested by jModeltest was unavailable. This model was applied individually to four partitions in the concatenated mtCO1-ITS dataset: one partition for each codon position in mtCO1, and a separate partition for ITS. Bayesian analyses were conducted in MrBayes (Huelsenbeck and Ronquist 2001, Ronquist and Huelsenbeck 2003), under the following conditions: 4 chains, 2 runs, and 1,000,000 generations for the COX1-ITS dataset. Each chain was sampled every 50 generations. The concatenated dataset was partitioned as in ML analyses, using the GTRgamma model for each of the three mtCO1 partitions and GTRinvgamma for the ITS partition. The LSU dataset was analyzed with 3,000,000 generations, under the GTRinvgamma substitution model. Maximum parsimony (MP) analyses were conducted in TNT (version 1.1), using TBR with 20 replications to find the best tree. Support for nodes was calculated via symmetric resampling (Goloboff et al. 2003). Trees were edited in FigTree (version 1.3.1) (Drummond and Rambaut 2007).

Table 4.3. PCR amplification and sequencing primers used in the study.

Primer	Sequence	Amplified gene	Reference
CO1F	5'-CCTACTATGATTGGTGGTTTTGGTAATTGAATAC-3'	mtCO1	Designed for this study by Steven M. Bogdanowicz
CO1R	5'-CAGGCAGTAAAATAAGCACGAGAATCTAAATCTAT-3'	mtCO1	Designed for this study by Steven M. Bogdanowicz
D2A	5’-ACAAGTACCGTGAGGGAAAGTTG-3’	LSU	Subbotin et al. 2006
D3B	5’-TCGGAAGGAACCAGCTACTA-3’	LSU	Subbotin et al. 2006
TW81	5’-GTTTCCGTAGGTGAACCTGC-3’	ITS	Subbotin et al. 2001
AB28	5-ATATGCTTAAGTTCAGCGGGT-3	ITS	Subbotin et al. 2001

4.3.3 Distinguishing strains of *D. siricidicola in silico*

Two specimens known to be *D. siricidicola*, "noc172", which is *D. siricidicola* Kamona, and "noc173," which is the North American strain of *D. siricidicola* were included to develop rapid diagnostic methods to distinguish Kamona from the North American strain. To distinguish strains of *D. siricidicola,* DNA sequences from the mtCO1 gene of nematodes were subjected to *in silico* enzyme digestion with CodonCode Aligner (version 3.7.1) to search for diagnostic restriction site patterns.

4.3.4 Identification of symbiotic fungal associates of *Sirex*

Because only female *Sirex* have mycangia containing *Amylostereum* fungus, there is no information on fungal identity for any of the male specimens included in the study. For eleven of the nematode specimens included in the study, isolation and characterization of the fungal associate of the host *Sirex* was conducted based on the intergenic spacer (IGS) region. The methods used to remove fungal symbionts, extract DNA, conduct PCR and sequencing, and analyze the results were the same as those used in Nielsen et al. (2009) for *A. chailletii* isolates, and fragment analysis was conducted to determine isolate of *A. areolatum* (Hajek et al., 2013).

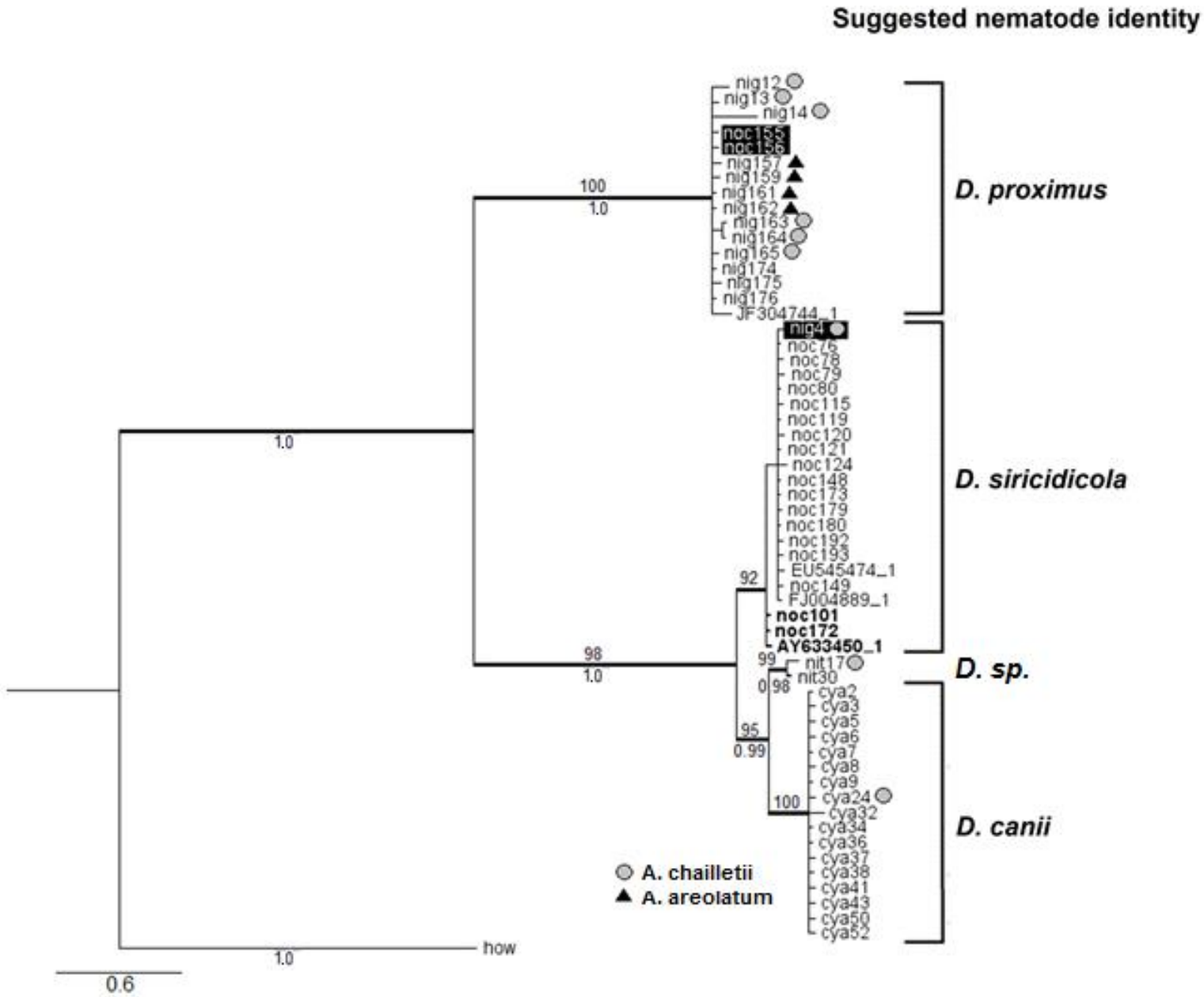
4.4 Results

4.4.1 Phylogenetic relationships among nematodes

Phylogenetic relationships resulting from the concatenated mtCO1-ITS dataset were largely congruent across all tree reconstruction algorithms. Since phylogenetic trees resulting from the LSU dataset were in conflict with the other two genes, this gene was excluded from further consideration. In general, there were four monophyletic clades of nematodes, mostly corresponding to *Sirex* host species (Fig. 4.1). Nematodes from *S. nigricornis*, *S. noctilio*, *S. cyaneus*, and *S. nitidus* each formed their own monophyletic clades.

There was strong support for host specificity in *Deladenus* species, except in three cases. Two samples from the *S. nigricornis* clade were found parasitizing *S. noctilio*. In another case, a sample from the *S. noctilio* clade was found parasitizing *S. nigricornis*. These exceptions are indicated by an asterisk (*) in Fig. 4.1.

Figure 4.1. Bayesian tree for *Deladenus* inferred from combined mtCO1 and ITS sequences. Values above branch points represent bootstrap support for clades well-supported by ML analysis (>70). Values below branch points represent the Bayesian posterior probability for well-supported clades (>0.95). Where MP analysis symmetric resampling values indicated well-supported clades (>75), branch lines are in bold. Branches without bold lines or numbers indicate the relationship was not supported above the aforementioned thresholds. *D. siricidicola* Kamona strain indicated in bold (noc101 and noc172). Suggested nematode species are indicated to the right of the clade. Instances of nematodes parasitizing unexpected *Sirex* hosts are highlighted in black. Known fungal associations of *Sirex* host are indicated with a circle (*A. chailletii*) or a triangle (*A. areolatum*). The outgroup is *Howardula aoronymphium*, a nematode parasitic on mycophagous *Drosophila*.


Suggested nematode identity
nig12
nig13
nig14
noc155
noc156
nig157
nig159
nig161
nig162
nig163
nig164
nig165
nig174
nig175
nig176
JF304744_1
nig4
noc76
noc78
noc79
noc80
noc115
noc119
noc120
noc121
noc124
noc148
noc173
noc179
noc180
noc192
noc193
EU545474_1
noc149
FJ004889_1
noc101
noc172
AY633450_1
nit17
nit30
cya2
cya3
cya5
cya6
cya7
cya8
cya9
cya24
cya32
cya34
cya36
cya37
cya38
cya41
cya43
cya50
cya52
how
100
1.0
1.0
98
1.0
92
99
0.98
95
0.99
100
1.0
0.6
D. proximus
D. siricidicola
D. sp.
D. canii
A. chailletii
A. areolatum

4.4.2 Distinguishing strains of *D. siricidicola in silico*

The combined presence of one AciI restriction site and three RsaI sites indicated the North American strain of *D. siricidicola*, whereas the absence of an AciI restriction site and the presence of at least one RsaI restriction site indicated *D. siricidicola* Kamona.
With the exception of noc172 and noc101 all *D. siricidicola* in the *S. noctilio* clade were the North American strain of *D. siricidicola*. This included the nematode specimen nig4, which was dissected from a *S. nigricornis*, indicating that in this instance the *S. nigricornis* was parasitized with the North American strain of *D. siricidicola*.

4.4.3 Identification of symbiotic fungal associates of *Sirex*

The *Amylostereum* fungus was identified to species from twelve of the fourteen *S. nigricornis* specimens from which nematodes were included in the study (Table 4.4). Four of the *S. nigricornis* carried *A. areolatum* in their mycangia, and five of the *S. nigricornis* carried *A. chailletii* in their mycangia. As the majority of *S. noctilio* included in the study were male, no successful fungal identification was possible. Of the two *S. nitidus* specimens included, only one was a female, and it was found to carry *A. chailletii* in its mycangia. Of the two nematode-parasitized *S. cyaneus* females from which fungus was identified, both carried *A. chailletii*.

Table 4.4. Species of symbiotic *Amylostereum* fungus associated with *Sirex* specimens included in the study.

Species of host *Sirex*	*Deladenus* specimen number	Species of *Amylostereum*	Isolate of *A. areolatum*	Genbank Accession No.
S. nigricornis	nig159	*A. areolatum*	BE	
S. nigricornis	nig161	*A. areolatum*	BE	
S. nigricornis	nig162	*A. areolatum*	BE	
S. nigricornis	nig157	*A. areolatum*	BE	
S. nigricornis	nig163	*A. chailletii*		
S. nigricornis	nig165	*A. chailletii*		
S. nigricornis	nig12	*A. chailletii*		KC411828
S. nigricornis	nig14	*A. chailletii*		KC411829
S. nigricornis	nig13	*A. chailletii*		KC411827
S. nitidus	nit17	*A. chailletii*		KC411826
S. cyaneus	cya24	*A. chailletii*		KC411830
S. cyaneus	cya52	*A. chailletii*		

4.5 Discussion

4.5.1 Nematode diversity and identification

For determining closely related strain of *Deladenus* in the present study, the two-gene concatenated dataset for mtCO1 and ITS provided the finest resolution. Phylogenetic analyses showed that, for the most part, each *Sirex* host species included in the study has a corresponding nematode. With one exception, *S. nigricornis* included in the present study all contained the same nematode genotype, which matched the *D. proximus* identified by Yu et al. (2011). Based on this information, we feel that the nematodes in our study collected from *S. nigricornis* are likely conspecific with those Bedding and Akhurst (1978) called *D. proximus* and collected in *S. nigricornis*.

Deladenus wilsoni is another possible name to apply to nematodes obtained from *S. nigricornis*. Morphologically, the two species would be difficult if not impossible to distinguish. However, Bedding and Akhurst (1978) reported that although *D. wilsoni* can parasitize some *Sirex* spp., it had not been found in *S. nigricornis.* Additionally *D. wilsoni* mostly parasitizes rhyssine wasps, which are parasitoids of *Sirex* species, and it rarely produces the parasitic stage when in the presence of *Sirex* larvae in nature (Bedding and Akhurst 1978). Future studies comparing gene sequences from nematodes found parasitizing rhyssines to nematodes parasitizing *S. nigricornis* could help elucidate the identities of these nematodes.

Nematodes infecting *S. cyaneus* likely represent *D. canii,* described from *S. cyaneus* in fir (*Abies*) in New Brunswick, Canada (Bedding 1974). *D. canii* also was said to be found in the southwestern United States (Bedding and Akhurst, 1978). However, it is likely that the

woodwasp referred to as *S. cyaneus* in the southwest was actually a different species. In fact, southwestern *Sirex* woodwasps were collected from a different genus of conifer (*Picea*) and were likely *Sirex nitidus* (H. Goulet, pers. comm.); therefore, it is possible that the nematode found in the southwestern *Sirex* was not the same nematode as was described from *S. cyaneus* in the eastern United States and Canada.

The sample noc172 is the biological control agent *D. siricidicola* Kamona. Based on the presence of restriction sites (with the exception of noc101), all nematodes found parasitizing *S. noctilio* were the non-sterilizing 'North American' strain of *D. siricidicola*. In some cases this was corroborated with data on whether the nematodes were present inside the eggs of the host or not (D.W.W., unpublished data). In many cases, however, this was difficult to diagnose, as many specimens originated from male *Sirex*, so there are no data regarding eggs. Moreover, the nematodes and their *Sirex* hosts had been stored in ethanol, which made it difficult to determine whether nematodes were inside of *Sirex* eggs or merely in the sheath of the egg as reported for the non-sterilizing strain of *D. siricidicola* by Yu et al. (2009). Yu et al. (2009) reported that the non-sterilizing strain of *D. siricidicola* is present in New York and Ontario. In the present study, the North American strain was found in New York as well as Pennsylvania. The sample noc101, which is the *D. siricidicola* Kamona strain, originated from a controlled release study (D.W.W., unpublished data). This distinction between the non-sterilizing 'North American' strain of *D. siricidicola* and *D. siricidicola* Kamona was also found by Leal et al. (2012), who reported that mtCO1 sequences for the two strains could be used to differentiate them. Additionally, they were able to develop a PCR-RFLP tool to differentiate the strains.

Linking the nematodes collected in the present study to those previously described from *Sirex* hosts (Bedding 1974; Bedding and Akhurst 1978) is challenging for several reasons. First,

none of the nematodes collected by Bedding and Akhurst (1978) were collected where our samples originated, so it is difficult to use geographic range as a guide. Second, the taxonomy of *Sirex* hosts from which the nematodes were collected has been in flux over the past several decades (Goulet 2012), making it difficult to link the *Sirex* hosts mentioned in Bedding and Akhurst (1978) to the *Sirex* hosts included in the present study. Moreover, it is difficult to obtain adults of these nematodes, which express the morphological characters key to species identification, as parasitized *Sirex* hosts contain only juvenile nematodes. Even if adults were available, using morphological characters to differentiate species of *Deladenus* may be problematic, however, as Chitambar (1991) stated that, among the species of *Deladenus* that have been identified as having an insect parasitic stage, morphological characters alone cannot be used to distinguish species. In fact, due to intergrading morphological characters, the nematodes could only be placed into one of two superspecies: the *D. wilsoni* superspecies, containing *D. wilsoni* and *D. proximus*; and the *D. siricidicola* superspecies, containing *D. siricidicola*, *D. canii*, *D. rudyii*, and *D. nevexii* (Chitambar 1992; Siddiqi 2000).

4.5.2 Host specificity and potential for non-target effects

The invasion of *S. noctilio* has led to new possible *Sirex-Deladenus* associations. Because *S. noctilio* and *S. nigricornis* can co-infest pine trees, there is potential for nematodes to switch hosts. Three samples from the present study appear to be cases of host switching. Among nematodes collected from *S. noctilio*, two specimens out of 19 were found to be the nematode more often associated with *S. nigricornis*. These samples, "noc155" and "noc156", were collected from Warrensburg, New York, where a number of *Deladenus* nematodes carried by *S.*

nigricornis specimens included in the study were collected. Likewise, among the nematodes collected from *S. nigricornis* specimens, one out of 14 was found to be the non-sterilizing strain of *D. siricidicola*. This sample, "nig4", was collected in Oswego, New York, where a number of *S. noctilio* containing the non-sterilizing strain of *D. siricidicola* were collected. This indicates a potential for non-target effects, should *D. siricidicola* Kamona be released in North America. It is not known, however, whether parasitization of *S. nigricornis* by *D. siricidicola* Kamona would lead to sterilization in *S. nigricornis*, nor is it known how frequently such a host switch might occur.

4.5.3 Host associations with *Amylostereum* species

With the exception of *D. wilsoni*, native North American *Deladenus* species have been thought to exclusively consume *Amylostereum chailletii* (Bedding and Akhurst 1978); however, four of the *S. nigricornis* specimens included in the study were found to carry *A. areolatum* in their mycangia (Table 4.4). Bedding and Akhurst (1978) stated that *Deladenus* nematodes (with the exception of *D. wilsoni*) are highly fungus-specific. Additionally, Chapter 2 describes differences in the reproductive output of the *D. siricidicola* Kamona strain when feeding on different isolates of *A. areolatum*. This suggests the possibility that nematodes associated with *S. nigricornis* are able to eat either *A. areolatum* or *A. chailletii*. If this nematode is indeed *D. proximus*, then this observation may not be too surprising, as *D. proximus* and *D. wilsoni* together comprise the *D. wilsoni* superspecies proposed by Chitambar (1991). Perhaps both nematodes in this superspecies are able to eat either species of *Amylostereum*. However, it could be possible that nematodes

found in these *S. nigricornis* specimens had access to *A. chailletii* also in the tree at the same time.

4.5.4 Usefulness of mtCO1, ITS, and LSU for distinguishing *Deladenus* nematodes

Yu et al. (2009) were able to distinguish the non-sterilizing strain of *D. siricidicola* from the *D. siricidicola* Kamona strain based on mtCO1 sequence data, and in the present study, mtCO1 also was useful for distinguishing among *Deladenus* nematodes. ITS has been useful for identifying species of entomopathogenic nematodes, as well as assessing their evolutionary history (Stock 2009). In the present study, MP analysis failed to resolve monophyletic clades based on ITS data, although the ML and Bayesian analyses both recovered well supported clades that reflect the two proposed superspecies. Ribosomal large subunit sequences (LSU) have been used to resolve taxonomic and phylogenetic issues at the genus and species level for nematodes, especially among *Steinernema* spp. (Stock et al. 2001). In the present study, no method of analysis was able to resolve clades based on LSU data (see supplementary materials), and resolution became worse with the inclusion of outgroup taxa in the genus *Howardula*. The two *Deladenus* superspecies proposed by Chitambar (1991) were supported in all three analyses of mtCO1; however, this locus offered poor resolution among nematodes collected from *S. noctilio*, *S. cyaneus*, and *S. nitidus.*

The native *Deladenus* fauna is poorly known, and this study has helped clarify *Sirex-Deladenus* associations in eastern North America. Understanding the native fauna and *Sirex*-nematode specificity is important for determining non-target effects, should *D. siricidicola* Kamona be introduced for biological control of *Sirex noctilio*. In particular, the apparent cross-

infectivity of nematodes typical of *S. nigricornis* and *S. noctilio* indicates a possibility for native *S. nigricornis* to become parasitized by the primary biological control agent of *S. noctilio*.

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Chapter 5

Trading Spaces: Fungus and Nematode Switch off as Predator and Prey[4]

5.1 Abstract

Use of the parasitic nematode *Deladenus siricidicola* to control invasive pine-killing *Sirex noctilio* woodwasps in the Southern Hemisphere is one of the most successful examples of classical biological control. The commercially available nematode parasitizes and ultimately sterilizes adult female woodwasps. Since the discovery of established *S. noctilio* in North America in 2005, the nematode is being considered for use for control in the United States, as the southern pine industry is potentially threatened. Both nematode and woodwasp rely on the white rot fungus, *Amylostereum areolatum,* for continued survival, and the nematode is commercially mass produced on this fungus. Previous studies have shown *A. areolatum* apparently parasitizing eggs and adults of *D. siricidicola*, so we tested a hypothesized a role reversal can occur wherein fungal hyphae invade and kill nematode eggs. *D. siricidicola* eggs were exposed to multiple isolates of *A. areolatum* to quantify the number of eggs lost to fungal invasion, *A. areolatum* and *A. chailletii* were observed via a combination of cryogenic scanning electron microscopy and fluorescence microscopy for their ability to parasitize both eggs and adults of *D. siricidicola* and *D. proximus*. This study reports the first evidence of a basidiomycete destroying nematode eggs,

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as well as a novel trapping mechanism used to capture and parasitize two species of adult female *Deladenus* nematodes.

Keywords: Nematophagous fungi, ecology, biological control, Basidiomycete, *Deladenus*, *Amylostereum*

5.2 Introduction

Many species of fungi, spanning Basidiomycota, Ascomycota, Zygomycota, and Chytridiomycota, have been found to have antagonistic relationships toward nematodes and their eggs, often using them for nutrition. Barron (1977, 1992) suggested that wood rot fungi, which are often in a nitrogen-limited environment, might make use of nematodes as a nitrogen supplement. Additionally, Barron suggested that because many fungi are parasitized by stylet-bearing fungal-feeding nematodes, these fungi might have evolved an antagonistic relationship towards such nematodes as a protective strategy.

The basidiomycete *Amylostereum areolatum* (Fries) Boidin (Russulales: Amylostereaceae) is a wood rot fungus integral to an intriguing ecological system. The fungus is necessary for the development of the invasive pine-killing woodwasp, *Sirex noctilio*, as well as the fungal food source for *S. noctilio*'s most successful biological control agent, the parasitic nematode *Deladenus siricidicola* Bedding (Tylenchida: Neotylenchidae) (Bedding 2009). Due to the invasive pest status of *S. noctilio* (Borchert et al. 2007), the role that *A. areolatum* plays for both the woodwasp and the nematode makes it important to elucidate the many individual interactions occurring between woodwasp, fungus, and nematode.

In adult female woodwasps, fragments of *A. areolatum* called ‘oidia’ or ‘arthrospores’ are carried within paired internal organs called ‘mycangia’ (Boros 1968). Oidia are injected into the pine tree during *S. noctilio* oviposition. The fungus is critical to *S. noctilio* in two main ways. First, phytotoxic venom is simultaneously injected into the tree with the fungus, and in concert, the fungus and venom cause lethal pine wilt of the tree (Coutts 1969). This allows *S. noctilio* larvae to develop unhindered by tree defenses. The second critical role played by *A. areolatum* is that early instar larvae require the fungus to develop to adults (Madden and Coutts, 1979). Larvae developing within the heartwood of the tree are unable to survive to adulthood in the absence of the fungus (Ryan and Hurley 2012).

A. areolatum is also required for the mass production of *Deladenus siricidicola* Kamona (Bedding 2009). This nematode has a dual life strategy, in which fungal feeding (mycophagous) nematodes in the tree feed exclusively on *A. areolatum*. In the presence of woodwasp larvae, however, the nematode switches its life strategy and develops into a parasitic form. Females of the parasitic form invade woodwasp larvae, eventually leading to sterilization of the *Sirex* hosts. A single generation of the parasitic nematodes is followed by the production of mycophagous generations (Bedding 2009; Bedding and Iede 2005). Mycophagous generations can carry on indefinitely, a trait which is exploited for the mass production of *D. siricidicola* Kamona.

Chapter 3 describes how *D. siricidicola* reproduction varied significantly with fungal isolate. In some cases, nematodes failed to lay many eggs on a given fungus, but in other cases, nematodes laid many eggs which were subsequently grown over by the fungal colony and failed to hatch. This study investigated the interactions between four pairs of *Amylostereum-Deladenus* isolates, to reflect a variety of associations. The ability of these *Amylostereum* species and isolates to invade their associated nematode’s eggs, juveniles, and adults was studied via

fluorescence microscopy. Cryogenic scanning electron microscopy was used to further examine an isolate of *A. areolatum* invading *D. siricidicola* Kamona. Additionally, cultures of two isolates of *A. areolatum* were inoculated with two strains of *D. siricidicola* eggs. The ability of these fungal isolates to grow over *Deladenus* eggs, with and without rich growing medium, was quantified, in order to estimate how much impact a given fungal isolate can have on nematode population growth.

5.3 Materials and Methods

5.3.1 Fungal cultures

To establish a fungal culture, a 3 mm diameter plug from the edge of a culture was transferred to a 90-mm diameter Petri dish containing half-strength potato dextrose agar (Difco, Sparks, MD) with a total of 25 g/L agar to make this medium harder (1/2PDAh) (R.A. Bedding, pers. comm.). The fungal cultures were incubated for 5 d at 23ºC in darkness prior to use in experiments.

5.3.2 Nematode strains

Four combinations of nematode and fungus were studied (Table 5.1). The Kamona strain of *D. siricidicola* used for studies was obtained in 2006 from Ecogrow Environment (Queanbeyan, N.S.W., Australia) where it is mass-produced for biological control of *S. noctilio*. *D. siricidicola* Kamona were kept under USDA, APHIS permit in a quarantine facility (the Sarkaria Arthropod

Table 5.1. Nematodes and fungi included in this study.

Nematode species	Nematode strain	Fungal isolates tested
Deladenus siricidicola	Kamona	*A. areolatum* BDF
	Non-sterilizing	*A. areolatum* BD
Deladenus proximus		*A. areolatum* BE
Deladenus sp. (undescribed)		*A. chailletii* G

Research Laboratory, Ithaca, New York) for the duration of the experiment. Nematodes were initially grown on *A. areolatum* BDF and were maintained inside brown paper bags in a 23ºC incubator with no light.

A colony of the non-sterilizing strain of *D. siricidicola* (NS) used for studies was established by transferring juvenile nematodes dissected from a single parasitized *S. noctilio* male and placing them on 1/3PDA plates previously inoculated with *A. areolatum* BD isolate. The host *S. noctilio* emerged from Scots Pine (*Pinus sylvestris*) collected from Tioga County in Pennsylvania in 2012. Nematodes were identified as the non-sterilizing strain of *D. siricidicola* via molecular characterization, as described in Kroll et al. (2013).

A colony of *D. proximus* used for these studies was established by transferring juvenile nematodes dissected from a single parasitized *S. nigricornis* male and placing them on 1/3PDA plates previously inoculated by *A. areolatum* BE isolate. The host *S. nigricornis* emerged from a Scots pine collected from Warren County, New York in 2012. Nematodes were identified via molecular and morphological characterization as described in Chapter 4.

A colony of an undescribed *Deladenus* was established by transferring juvenile nematodes dissected from a single parasitized *S. californicus* male and placing them on 1/3PDA plates previously inoculated by *A. chailletii*. The host *S. californicus* emerged from a *Pinus moniticola* X *P. strobus* hybrid tree collected by Jenni Cena from King County, Washington State in 2009.

5.3.2 Egg survival assay

Two experiments were conducted: one to test the ability of *A. areolatum* BD isolate to prevent the hatching of a non-sterilizing strain of *D. siricidicola* eggs, and one to test the ability of *A.*

areolatum BDF isolate to prevent the hatching of *D. siricidicola* Kamona eggs. For nematode growth assays, there were three treatments. Two treatments included *A. areolatum* fungus, growing either fast (on 1/2PDAh medium) or more slowly (water agar). The control treatment did not include fungus, only 1/2PDAh medium. Assays were conducted at 23^{o}C in darkness. To inoculate the treatment plates with eggs, nematode colonies were flooded with distilled water and the liquid containing nematodes and eggs was filtered once through a Swinnex filter holder (Millipore) equipped with a 60 µm filter, which allowed eggs to pass through into the filtrate. The concentration of eggs in the stock suspension was estimated by counting all eggs from each of ten 20 µL samples under a dissecting microscope at 20X magnification. The solution was diluted with sterile, distilled water to contain 500 eggs per 20 µL drop. One drop was added to each of 15 cultures of *A. areolatum* at the growing margin of the fungal colony or, in the case of the control treatment, 1 cm from the center of the plate. Three plates per treatment were destructively sampled every 24 h to quantify the number of eggs and juveniles per plate. For each sampling period, dishes were flooded with water and ten 20 µL samples of each of the washings were examined under a dissecting microscope to volumetrically determine the total number of eggs and living nematodes per dish. Each of the two experiments was repeated for a total of three experimental replicates per nematode/fungus combination.

5.3.3 Microscopy

Four nematode-fungal combinations were tested (Table 5.1). For each combination, fungal cultures were established and inoculated with nematodes as above. Nematode colonies were grown at 23 ºC for 14 d. Between 14 and 25 d, colonies were examined with a dissecting

microscope, and eggs and nematodes at different infection stages were removed with a minutin pin. Such samples were either mounted in a drop of water on a slide with a cover slip for direct viewing at 100 – 400 X magnification under light microscopy, or stained for fluorescence microscopy.

To stain fungal hyphae for fluorescence, sample nematodes were placed on a microscope slide in a drop of Calcofluor/Evans Blue (1.0%, 0.5%). A drop of 1M potassium hydroxide was added, and a coverslip was placed on top. Slides were then examined under fluorescence microscopy using an excitation wavelength of 440 nm. Both types of slides were examined to observe fungal infection. Additionally, a sterile scalpel was used to excise agar medium containing fungus and nematodes, and these excised pieces were placed on a slide and viewed via light microscopy at 100 – 400X magnification.

5.3.4 Selective staining of fungal cystidia

To confirm the role of cystidia, which do not stain with Calcofluor, some nematodes exhibiting fungal parasitism were removed with a minuten pin. These samples were placed on a slide in a drop of aqueous safranin solution (1% w/v), which stains cystidia deeply (Talbot, 1964). A cover slip was added, and samples were examined under a compound microscope.

5.3.5 Cryogenic scanning electron microscopy

D. siricidicola Kamona colonies were established as above on 90 mm Petri dishes, with one modification. Prior to fungal inoculation, autoclaved cellophane was placed on the 1/2PDAh

medium. Colonies were kept at 23ºC for 2 weeks, at which time they were observed under a dissecting microscope (20X magnification) for signs of fungal parasitism. A scalpel was used to excise 1 cm X 0.5 cm rectangles of cellophane containing fungus and nematodes. Samples were quickly attached to brass stubs using TissueTek® and frozen at -80 ºC in liquid nitrogen for up to 2 weeks until they were transferred to the cryostage of the SEM (Hitachi 4500) for viewing.

5.4 Statistical Analysis

5.4.1 Egg survival assays

The total numbers of juveniles observed per day were log10 transformed (x + 1) and analyzed using a two-way ANOVA with treatment, day, and the interaction between treatment and day as main effects. Experimental replicate was coded as a random effect. Means were separated with Tukey's LSM test (JMP 2013).

5.5 Results

5.5.1 Egg survival assay: *D. siricidicola* Kamona vs. *A. areolatum* BDF

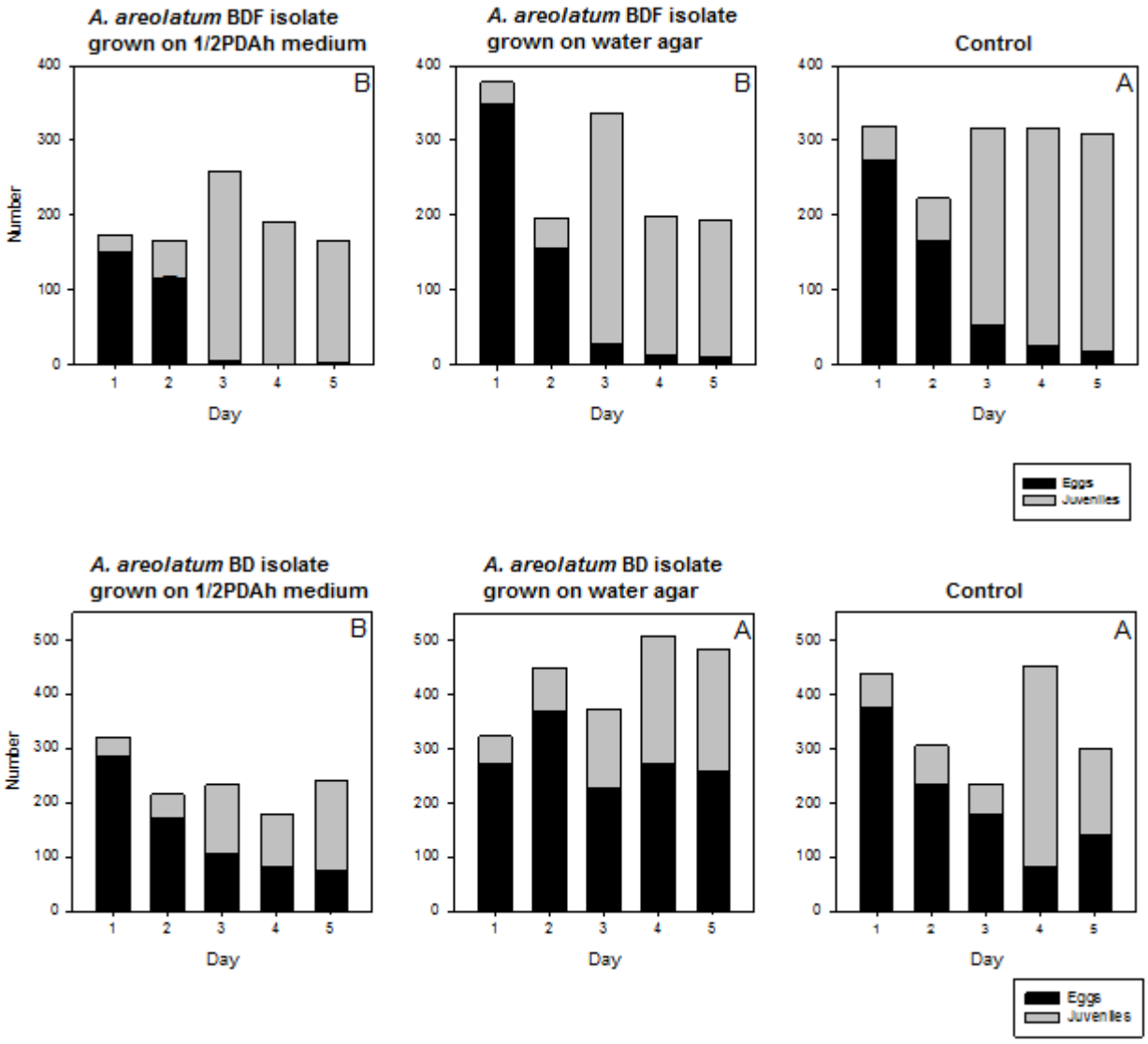
Both day and treatment had significant effects on the total *D. siricidicola* Kamona juveniles produced ($F_{1,127}$ = 241.31; $p<0.0001$; $F_{2,127}$ = 9.68; p = 0.0001), respectively. *A. areolatum* BDF isolate grown on 1/2PDAh medium and grown on water agar produced significantly fewer juveniles (115.7 ± 11.2; 128.9 ± 13.7, respectively) (mean ± SE) than the control treatment of

1/2PDAh only (189.8 ± 18.6) (Fig. 5.1A). There was no interaction between treatment and day ($F_{2, 127}$= 0.39; p = 0.68).

5.5.2 Egg survival assay: Non-sterilizing strain vs. *A. areolatum* BD

Both day and treatment had a significant effect on the total non-sterilizing *D. siricidicola* juveniles produced ($F_{1,127}$ = 165.53; p < 0.0001; $F_{2,127}$, = 10.20; p < 0.0001), respectively. *A. areolatum* BD isolate grown on 1/2PDAh medium produced significantly fewer juveniles, with an average of 89.4 ± 8.9 juveniles (mean ± SE), when compared with either *A. areolatum* BD grown on water agar (133.4 ± 13.4) or the control treatment of 1/2PDAh only (132.0 ± 15.0) (Fig. 5.1B). There was no interaction between isolate and day ($F_{2, 127}$= 1.02; p = 0.36).

Figure 5.1. Mean numbers of *D. siricidicola* juveniles and eggs after 5 d when grown on *A. areolatum* BDF and *A. areolatum* BD grown on 1/2PDAh medium, water agar, or with no fungus. Upper case letters denote significant differences in the total juveniles over all days combined



5.5.3 Microscopy

Cryogenic scanning electron microscopy showed *D. siricidicola* Kamona eggs overgrown by *A. areolatum* BDF isolate (Fig. 5.2A), as well as the apparent penetration of an egg by a hyphal tip (Fig. 5.2B). Light microscopy revealed piles of melanized nematode eggs overgrown by fungus (Fig. 5.2C), while fluorescence microscopy showed fungus growing inside of eggs (Fig. 5.2D). This was observed for all *Deladenus-Amylostereum* combinations tested.

Additionally, adult female nematodes were found parasitized by fungal hyphae for all *Deladenus-Amylostereum* combinations tested (Fig. 5.3A-D). Hyphae always invaded adult females via the vulva, and nematodes were frequently found thrashing vigorously despite fungal invasion. Fungal-parasitized nematodes were tethered to the substrate via fungal cystidia, which attached to the nematode vulva at one end, and grew into the substrate at the other end. Fluorescence microscopy revealed extensive hyphal growth within nematode bodies (Fig. 5.3B, D), which eventually killed the nematode and grew out from the cuticle along the length of the body. Cystidia in fungal cultures stained deeply red in safranin, as did the cystidia tethering parasitized nematodes to the substrate (Fig. 5.3C).

Figure 5.2. *Deladenus* eggs attacked by *Amylostereum areolatum*. A. *D. siricidicola* Kamona eggs attacked by *A. areolatum* BDF isolate, cryogenic scanning electron microscopy. B. Cryogenic scanning electron microscopy of a *D. siricidicola* Kamona egg, apparently penetrated by *A. areolatum* BDF isolate hyphal tip (arrow). C. Darkened *D. proximus* eggs attacked by *A. areolatum* BE isolate. D. Assimilative hyphae of *A. areolatum* BE isolate inside of a *D. proximus* egg, under fluorescence staining. Images in the same column share the same size bar.

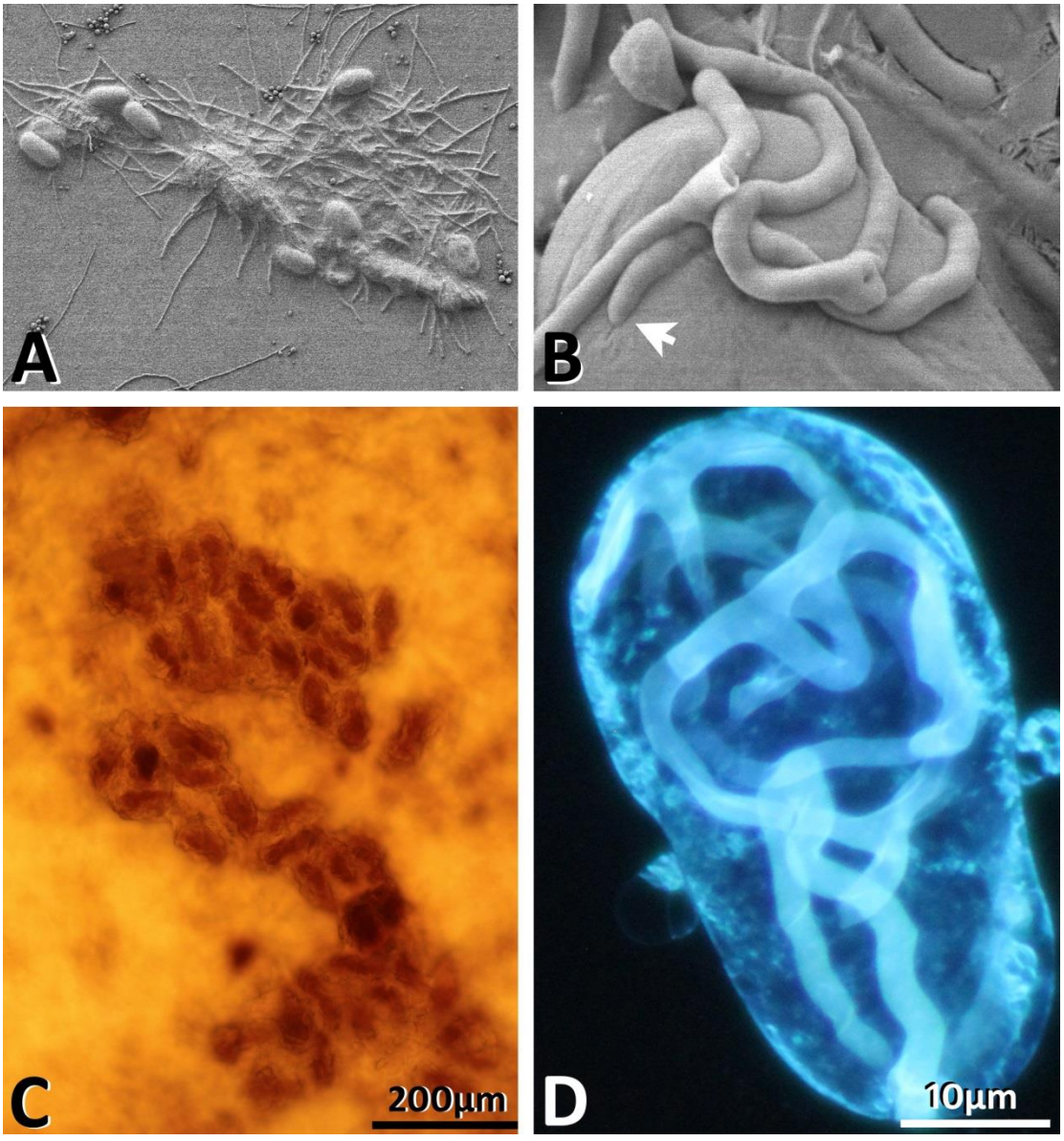
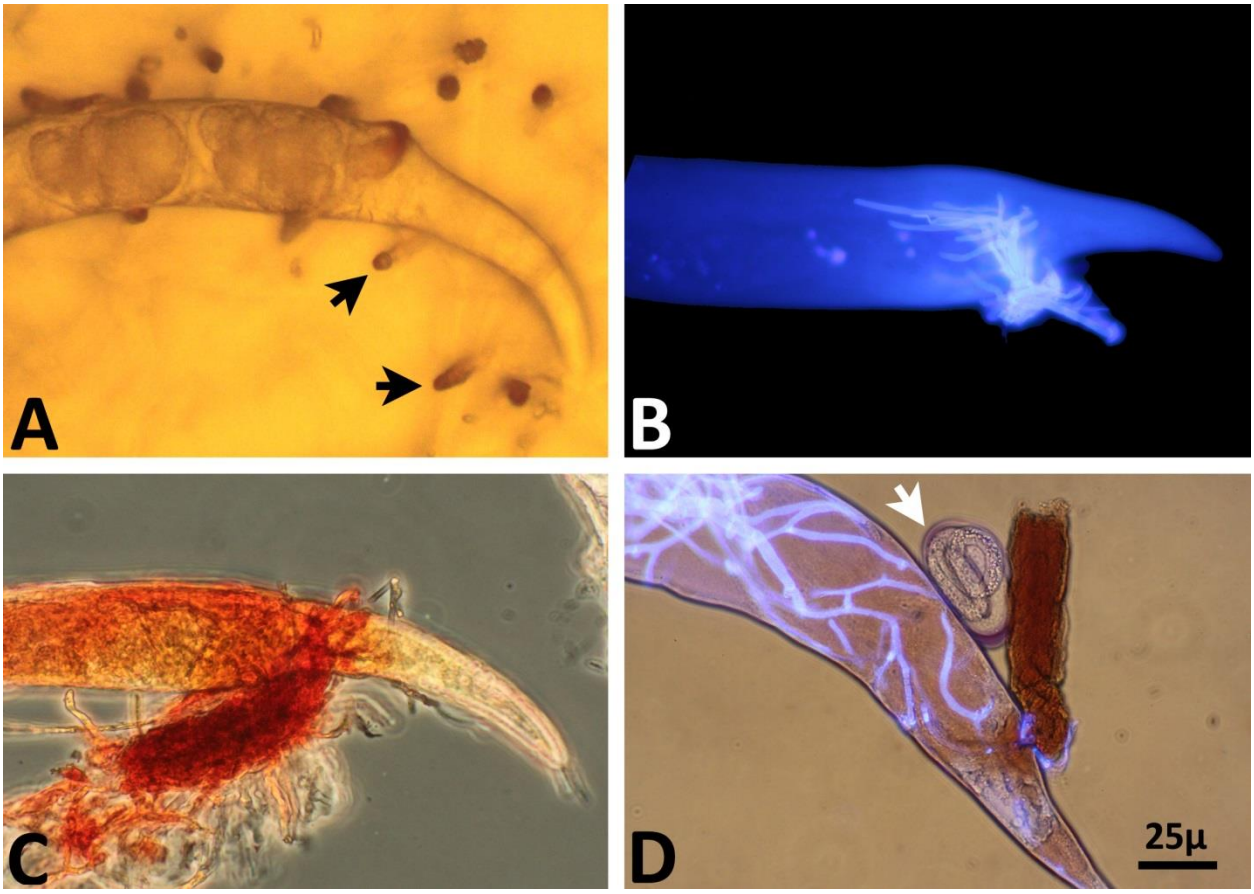


Figure 5.3. Parasitized adult female *Deladenus* vulvas. A. *D. siricidicola* NS strain nematode surrounded by cystidia (black arrows), early stage of infection. B. Undescribed *Deladenus* species parasitized by *A. chailletii* hyphae. C. Safranin staining of *A. areolatum* BD isolate cystidium penetrating adult female *D. siricidicola* NS strain vulva. D. Fluorescence staining showing internal hyphae of *A. areolatum* BD isolate within adult female nematode body. White arrow indicates nematode egg.



5.6 Discussion

Previously, it has been noted that to culture *D. siricidicola* on *A. areolatum*, a "balance must be maintained between nematodes and fungus since the nematodes can breed successfully only on the growing edge of the fungus." (Bedding 1972). The results of this study provide insight into what drives the balance between *Amylostereum* fungi and *Deladenus* nematodes; namely, that the fungus can kill and consume both adult nematodes and eggs. These results help explain suppression of reproductive output of both *D. siricidicola* and *D. proximus* when grown on different species and isolates of *Amylostereum* fungus (Chapter 3).

Nematophagy occurs across major groups of fungi and is believed to have evolved multiple times (Barron 1992). The three established categories of nematophagous fungi are predatory (nematode-trapping), endoparasitic, and egg-parasitic (Lopez-Llorca and Jansson, 2007). Due to the low nitrogen availability in wood, it has been hypothesized that wood rot fungi like *Amylostereum* would be particularly suited to nematophagy, especially when the nematode prey is a fungal grazer (Barron 1977). The present study supports this hypothesis. All combinations of *Amylostereum* and *Deladenus* tested showed evidence of parasitism of adult female nematodes. The fungal entry route consistently occurred via the vulva, and frequently fungal invasion was observed in a live, vigorous host. Moreover, *A. areolatum* BDF and BD isolates both showed the ability to kill and invade nematode eggs. This ability of *Amylostereum* to invade *Deladenus* is novel among basidiomycetes in its method of capture of adult nematodes and its ability to kill nematode eggs.

While nematode-destroying fungi have been found in many major groups, including Basidiomycetes, Ascomycetes, Zygomycetes, and Chytrids, fungi parasitizing nematode eggs

have only been described from Ascomycetes and Zygomycetes (Lopez-Llorca and Jansson, 2007). These fungi either impact nematode eggs directly, by penetration, or indirectly, by distorting embryos. Prior literature mostly focuses on the direct impact of fungi penetrating root knot nematode cysts (Lopez-Llorca and Jansson, 2007), with little attention paid to indirect impact. However, it is known that *Phoma* species (Ascomycetes) are able to produce pigmented diffusible metabolites which penetrate nematode egg shells, leading to failure to hatch (Rodríguez-Kábana and Morgan-Jones, 1988). The present study set out to characterize the nematode egg destruction exhibited by *A. areolatum*. While hyphae frequently grew over eggs which then darkened and did not hatch, cryogenic scanning electron microscopy did not reveal any apparent formation of appressoria. However, with fluorescence, extensive assimilative hyphae could be found inside nematode eggs. Prior literature on nematode-destroying fungi suggests this method of egg destruction would be labeled as "indirect"; however, the plant pathology term "necrotrophic" seems more appropriate. A parasitic relationship that is necrotrophic is defined as one in which the organism derives energy from killed cells (Lewis 1973).

Nematophagous basidiomycetes employ numerous methods of prey capture, including traps, adhesive nets, and special spore structures (Lopez-Llorca and Jansson, 2007). Although *A. areolatum* forms asexual arthrospores in culture (Talbot, 1964), these were never observed playing a role in nematode infection. However, another structure formed by both *A. areolatum* and *A. chailletii*, the encrusted cystidium (Talbot, 1964), does appear to play a role in nematode infection. Cystidia tethered parasitized adult female nematodes by the vulva to the substrate, suggesting a novel trapping method. The function of cystidia in basidiomycetes is largely unknown, although some studies suggest they may provide protection from fungal grazers.

Nakamori and Suzuki (2007) found cystidia of the fruit bodies of *Russula bella* and *Strobilurus ohshimae* increased collembolan mortality in lab studies, and decreased the number of Collembola. Barron and Dierkes (1977) found cystidia of *Hohenbuehelia* fungi bearing hourglass or sphericial secretory cells at their tips, although they could not demonstrate a nematode-capturing ability of these cells. For some nematophagous fungi, trap adhesives are activated upon contact with nematode cuticle, a response which is thought to be mediated by lectin binding (Nordbring-Hertz and Mattiasson, 1979). However, in the present study, the mechanism by which the attachment occurs remains unknown.

Another notable difference between the antagonistic relationship between *Amylostereum* fungi and *Deladenus* nematodes when compared with other known nematophagous fungi is the ecosystem in which the antagonistic relationship occurs. Most nematophagous fungi have been described from soil habitats (Barron 1977; Lopez-Llorca and Jansson, 2007), whereas *Amylostereum* and *Deladenus* occur within a tree. One exception is *Esteya vermicola*, an Ascomycete that preys on the pinewood nematode, *Bursaphelenchus xylophilus* (Wang et al., 2008; Wang et al., 2011; Liou et al., 1999). It is possible that *Amylostereum* fungi, growing in a dearth of nitrogen, could benefit from using nematodes as an additional source of nitrogen. The role of nitrogen in nematophagy has been studied (Barron 1992) with nitrogen-limiting conditions leading to increased nematode consumption. Another possible benefit to consuming *Deladenus* nematodes emerges when one considers the relationship between *Sirex* woodwasps and their *Amylostereum* fungal symbionts. For example, *A. areolatum* is rarely observed to make fruiting bodies in the field, and the clonal nature of this fungus suggests it relies on ovipositing *S. noctilio* to get to a new *Pinus* host (Thomsen and Koch, 1999). Given that *D. siricidicola* parasitism of *S. noctilio* can have a negative impact on *S. noctilio*, either by leading to

sterilization or by reducing woodwasp size (Bedding 2009; Kroll et al., 2013), it would benefit *A. areolatum* to reduce nematodes able to parasitize *S. noctilio*, its vector.

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